ADVANCES IN PATHOGENESIS, ETIOLOGY, AND THERAPIES FOR ANKYLOSING SPONDYLITIS

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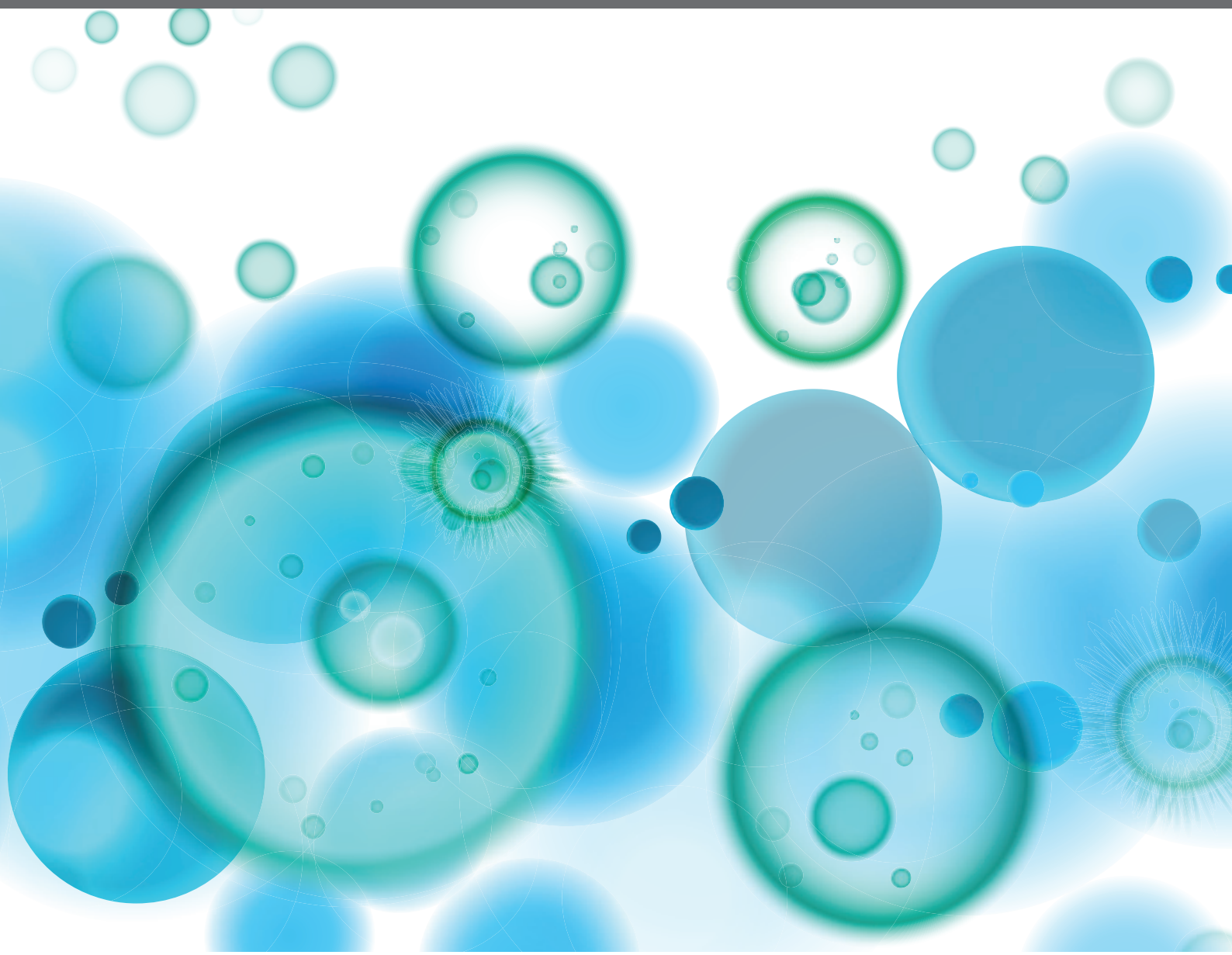

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ADVANCES IN PATHOGENESIS, ETIOLOGY, AND THERAPIES FOR ANKYLOSING SPONDYLITIS

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Editorial: Advances in Pathogenesis, Etiology, and Therapies for Ankylosing Spondylitis

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Keywords: ankylosing spondylitis, acquired immunity, diagnosis, innate immunity, therapy

Editorial on the Research Topic

Advances in Pathogenesis, Etiology, and Therapies for Ankylosing Spondylitis

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Ankylosing spondylitis (AS) is a chronic inflammatory rheumatic disease caused by the disrupted balance of both the innate immune system and acquired immune system in response to environmental factors (1). AS causes inflammatory back pain and affects the spine and sacroiliac joints, which can lead to a drop in life quality of patients, as well as an increased burden to patients and society (2). In recent years, a growing number of studies have been conducted to investigate the pathogenesis and etiology, imaging techniques, and treatment in AS (3–7). In this Research Topic, there are three review articles and seven research articles published, mainly focusing on pathogenesis and etiology, diagnosis and therapies, and related assessment tools of AS.

Hong et al. investigated the genetic association between IL 6 and autoimmune arthritis using multiple genome-wide association studies (GWAS) datasets, and they found a genetic association between the increased level of IL-6 signalling and risk of RA and AS, as well as observed the sexual difference in IL6-intermediate susceptibility to autoimmune arthritis. Controversial results on the effect of infections on the risk of AS were reported by previous studies, which is quantitatively investigated by Zhang et al. through a meta-analysis. They confirmed that the risk of AS can be significantly enhanced by infections, such as infections with adjusted comorbidities, viral infection. Liu et al. further conducted a meta-analysis to clarify the alteration of the immune system in patients with AS. They found that the pathogenesis of AS can be ascribed to the disequilibrium between Th17 and Tregs, Th1 and Th2, which further supports that AS is resulted from the disrupted balance of the innate immune system and acquired immune system (8).

The diagnosis is the key to reducing the burden on patients and society caused by AS. Tu et al. employed the MRI images to identify non-radiographic axial spondyloarthritis (nr-axSpA) and radiographic axial spondyloarthritis (r-axSpA), the latter of which is also known as AS. They found that AS patients presented more active inflammatory and chronic structural damages, while erosion was more frequently observed in MRI of nr-axSpA patients. Han et al. systematically analysed clinical and imaging hip data to examine hip changes in AS patients by MRI and X-ray. They observed that more than 40% of AS patients with minimal or no hip pain had hip changes, which can be used for early diagnosis.

Various therapies for AS have been studied, such as tofacitinib – an oral Janus kinase inhibitor (9) and Risankizumab – an IL-23 inhibitor (10). An increasing number of studies found that cytokine signalling *via* the IL-17A pathway was a major factor in the pathogenesis of spondyloarthritis (SpA) (11, 12). Tok et al. assessed the influence of inhibition of RAR related orphan receptor-g (RORC) on experimental SpA in HLA-B27 transgenic (tg) rats, and they found that experimental SpA in the HLA-B27 tg rat model could be accelerated and aggravated by RORC inhibitor treatment. Wang and Maksymowych reviewed recent studies on the role of the IL-23/IL-17 pathway in the pathophysiology of inflammation to discuss the treatment of AS. They found that the inhibition of IL-17 cytokines contributed to the inflammatory symptom control in patients with axSpA, but the IL-23 blockade was ineffective in the treatment. Chen et al. employed the gut microbiome as a biomarker to evaluate the effectiveness of adalimumab therapy in AS patients, based on previous discoveries that the gut microbiome was associated with the initiation and development of AS, and their findings suggested the gut microbiome was restored by adalimumab therapy in AS patients and therefore could be used as a predictive tool for treatment response.

Moreover, assessment technologies have been investigated to support the diagnosis and therapeutic interventions. Han et al. studied the effect of therapeutic interventions that can be assessed by Micro-CT, and they found that Micro-CT could be used to quantitatively assess the extent of axial involvement in mouse spondylitis caused by proteoglycan aggrecan (PGA). Zhang et al. quantitatively assessed the effect of tumour necrosis factor (TNF)-a inhibitor treatment in patients with spondyloarthritis (SpA) through clinical and MRI assessment, which was suggested as an accurate evaluation tool to guide targeted treatment.

In conclusion, the papers collected in this Research Topic contributed to the understanding of the pathogenesis and etiology of AS, including hereditary and environmental factors, as well as the development of diagnosis and therapies, and related assessment technologies.

AUTHOR CONTRIBUTIONS

C-WC and JC-CW contributed equally to the writing and reviewing of the article. All authors contributed to the article and approved the submitted version.

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A Comprehensive Assessment of Hip Damage in Ankylosing Spondylitis, Especially Early Features

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Ankylosing spondylitis (AS) is most common in adolescents and the ultimate result is disability, which places a huge burden on patients and society. Therefore, the key to improve the prognosis of AS is the early diagnosis of hip injury. To examine if AS patients whose hip pain is either absent or minimal might already have observable MRI and X-ray hip changes. Clinical and imaging hip data were systematically analyzed in 200 healthy controls (HC) and 300 AS with varying degrees of hip pain. Forty-four patients with early hip osteoarthritis (OA) served as positive imaging controls. In MRI images, BME lesions in the STIR sequence were much more frequent in AS (62%) compared to HC (2%) ($p < 0.0001$). Most importantly, 42% of AS with no or minimal hip pain had one or more MRI lesions. This was much more frequent compared to the 2% in HC ($p < 0.05$). These lesions in AS were observed singly or in combination in the trochanters (8%), femoral heads (12%), and acetabula (13%). Parallel finding that X-ray changes were present in patients with minimal or no hip pain was also observed with X-ray. Based on the normal hip width of HC, joint space narrowing was observed in 94.3% of the entire AS cohort, and importantly 56.7% of AS patients with no or mild hip pain. In these latter patients, functional activities of the hips such as walking were normal. At least 40% of AS patients with minimal or no hip pain might already show MRI and X-ray changes.

Keywords: ankylosing spondylitis, hip, MRI, X-ray, Harris score

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INTRODUCTION

Axial spondyloarthritis consists of two groups of entities, ankylosing spondylitis (AS), also known as radiographic axial spondyloarthritis, and non-radiographic axial spondyloarthritis (nr-axSpA). Radiographic sacroiliitis is present in AS but not in nr-axSpA (1). In the majority of ethnic groups, many AS patients carry the HLA-B27 gene (1–3). Other than pain, the two most common disabling features are bridging of vertebral syndesmophytes and hip destruction (4–6). The disease activity in many patients can be controlled by a TNF inhibitor, an IL-17 inhibitor, or a Janus kinase inhibitor (4, 7, 8). Not all patients develop syndesmophytes or hip destruction (9). It is commonly thought that early treatment can prevent syndesmophyte formation (10, 11). However, prevention of

progression has not been studied for hip destruction. Such studies need markers that might identify early hip disease.

Hip lesions have been reported in 25 to 35 percent of AS patients (12, 13). The typical symptom is groin pain. As a whole, hip lesions may be more severe in patients with early age onset, highly active axial, and entheseal diseases (14, 15). Very few imaging studies have been done with the aim of identifying early hip lesions. By the time a patient develops even moderate hip pain, the destructions are usually extensive (12). By that time, due to the lack of alternate treatment, hip arthroplasty is often the only effective therapy (12). Almost 90 percent of patients will experience pain relief and improved ranges of motion from hip arthroplasty. Ninety percent survived the replaced hip for 10 years, and 72 percent for 15 years (16, 17). Nevertheless, in theory, a patient would prefer using medications to arrest the progression of destruction before it requires arthroplasty.

One of the major objectives of this project is to examine if patients with minimal or no hip pain might already have X-ray and MRI changes. Investigators can then test if the progression of arthritis can be arrested by more aggressive medical therapies.

METHODS

Study Design and Participants

Two hundred healthy control (HC), 300 outpatients with AS with or without hip pain, and 44 patients with early OA of hips were recruited from the clinics of the Department of Clinical Rheumatology at Xijing Hospital, Xi'an, China. All AS patients fulfilled the 1984 modified New York criteria. Their spondylitis was regarded as being clinically active. None of them were on biologics at the time of evaluation. We follow three steps. The first step is to identify among a list of clinical parameters, the one which by itself is most useful for assessing hip involvement. The second is to identify X-ray parameters that might appear in early hip involvement. The third is to identify corresponding MRI changes. As for comparisons, we also investigate patients with moderate and severe hip pain. For positive controls, we used early hip osteoarthritis.

None of AS patients had experienced hip injury or showed non-Spondyloarthritis causes of hip arthritis. Patient with psoriasis or inflammatory bowel disease were excluded from the study. The study and the informed consent have been approved by the ethics committee of the Xijing Hospital of Fourth Military Medical University (ID: 20110303-7).

Radiography

X-rays were taken with subjects in the supine position with the targeted hip(s) at the center of focus. They were instructed to extend their lower limbs, and to rotate them so that the two big toes would touch one another. The upper margin of the image included the superior iliac crests. The lower margin included the upper third of the femurs. Quantitative hip joint width (HJW) measurements were assessed on images in a DICOM viewer. Three sites were measured: superomedial, superolateral, and the point of narrowest part of each hip space. For each patient, the narrowest side of the three measurements was used for comparison to other patients. The team of readers consisted of two radiologists and two rheumatologists. Readers were blinded to the clinical data. In our own cohort, the X-rays of the hips of OA patients were assessed systematically for the following three features: osteophytes, subchondral sclerosis and femoral head deformity.

MRI

MRI examinations were performed using standard protocols for T1W1 and STIR sequences using a 1.5-T machine (Magnetom Aera; Siemens Medical Solutions, Erlangen, Germany). Patients were placed supine with the hip joint in a neutral position. The parameters for T1-weighted images were TR/TE 715/9.5ms, for coronal short-tau inversion recovery (STIR), repetition time was 3550, echo time 51, and inversion time 145ms. Slice thickness was 4 mm. Both hips were included in the same image. Formal readings were preceded by a learning session. In the learning session, MRIs of a randomly selected subset of subjects were read separately by the four experienced readers. We divided the intense STIR signals into the following categories: subchondral acetabular, subchondral femoral head, cysts in acetabula, cysts in the femoral heads, enthesitis at greater trochanter, enthesitis at lesser trochanter. The readers were blinded to the clinical data. Afterwards, the four readers reviewed their scores together and discussed about the discrepancies. This learning session was followed by formal reading of all MRI. Disparities were reviewed together to arrive at a consensus.

Statistical Analyses

Analyses were performed using the SPSS 19.0 software (IBM, Armonk, NY, USA). Because the distribution was non-parametric, the Mann-Whitney U test was used for intra-group comparisons. In descriptive analysis, quantitative parameters were expressed as means and standard deviations. Qualitative parameters were expressed as percentages. Principal component analysis (PCA) was used to assess the percent contribution of each of different parameter. Kruskal-Wallis test was used for comparing samples of different sample sizes. Spearman's rank correlation test was used to evaluate the degree of correlation. The threshold statistic significance was set at $p < 0.05$.

RESULTS

Demographic

In **Table 1**, we show the demographic of the 300 AS patients, 44 OA patients and 200 healthy control. Eighty six percent of AS patients were HLA-B27 positive. The mean age of the AS group is not statistically different from the healthy control but less than the OA group ($p < 0.05$ comparing mean ages between AS and OA).

Identifying the Most Useful Clinical Parameter to Assess Hip Involvement in AS

We first used the parameters in the Harris Score as a clinical tool to assess hip joint disease activity and function. These parameters

TABLE 1 | Demographic features and pretreatment values for evaluation parameters of groups.

Characteristics (mean ± SD or %)	HC group	OA group	AS group
number of individuals	200	44	300
Age in years	29.9 ± 10.45	69.9 ± 5.54	25.8 ± 10.32
Male	73%	54%	82%
Duration (Month)	NA	NA	67.38 ± 65.39
Harris Hip Score (100 = best health)	94.49 ± 6.59	68.4 ± 6.33	77.82 ± 8.82
Hip pain score (44 = best health)	0	39.19 ± 6.65	24.95 ± 5.57
Flexion (degree)	132.8 ± 16.17	129.1 ± 22.3	125.31 ± 2.67
Abduction (degree)	29.16 ± 6.62	28.24 ± 6.15	26.19 ± 6.62
Extorsion (degree)	25.62 ± 0.61	25.5 ± 3.45	13.61 ± 6.61
Adduction (degree)	24.82 ± 2.46	22.5 ± 3.43	23.8 ± 5.45
Walking distance (m)	>1000	634 ± 256.6	521.2 ± 341.8
Function (0 = normal)	NA	32.78 ± 6.12	38.05 ± 5.11
BASDAI (0-10)	NA	NA	5.69 ± 1.58
BASFI (0-100)	NA	NA	37.48 ± 18.78
BASMI (0-10)	NA	NA	1.26 ± 1.98
Patient Global (0-10)	NA	NA	8.02 ± 1.25
CRP (higher than normal)	NA	NA	71%
ESR (higher than normal)	NA	NA	78%
HLA-B27 positive	NA	NA	86%

HC, Health control; OA, Osteoarthritis; AS, Ankylosing Spondylitis; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; BASFI, Bath Ankylosing Spondylitis Functional Index; BASMI, Bath Ankylosing Spondylitis Metrology Index; CRP, C-reactive protein (normal <0.8mg/dl); ESR, Erythrocyte Sedimentation Rate (normal Male<15mm/h, Female<20mm/h). Numbers are in means ± SD. NA, not available.

included hip pain, three separate hip ranges of motion (flexion, external and internal rotation), function, and walking distance. The Harris scores of both AS (77.5 ± 8.7) and OA (81.2 ± 6.4) were significantly lower than those of healthy normal controls (94.1 ± 3.6) ($p < 0.0001$). In AS, correlation analysis showed that among the variables in the Harris score, a strong correlation was observed only between pain and walking distance ($r = 0.7$, $p < 0.0001$). This showed that each parameter was relatively independent of the others. In AS, Harris score did not correlate with age, disease duration, BASDAI, BASMI, BASFI, ASDAS, CRP or ESR ($p > 0.5$). The lack of correlation to those parameters indicated that for AS, the Harris score was an independent evaluation tool. We used PCA to calculate the percent of contribution of each of the variable within the Harris score to the final score. PCA showed that pain accounted for at least 50% of the total variables. The contribution of the other parameters was relatively even and much less than that from pain. Harris Score divided the intensity of pain into three categories: minimal/none, moderate and severe. For hip pain, in our cohort, 33% of patients reported minimal/no pain, while 28% reported moderate pain, and 39% severe pain. In the OA cohort, 73% reported minimal/no pain, while 21% reported moderate pain, and 6% severe pain.

Positive Controls for X-ray and MRI Changes Using OA Images

Results are of X-ray features of the hips were tabulated in **Table 2**. In the 200 subjects in the HC group, osteophytes subchondral sclerosis and femoral head deformity were present in 1%, 2% and 0% respectively. For the 44 OA patients, those features were much more frequently at 29%, 87% and 32% respectively (p values of each ranged from < 0.001 to <0.0001). The joint width of hips in our OA cohort varied considerably. The mean and SD were 3.2 ± 0.7 mm. The mean was less than that of the 4.8 ± 0.74 mm of HC ($p < 0.001$). When analyzed by regression analysis, there was a small degree of correlation between the joint width and degree of pain ($r = 0.408$, $p = 0.006$). As expected, the mean joint width was the smallest in the group with severe pain.

We systematically evaluated the STIR images of the OA hips for BME lesions in trochanters, superficial subchondral BME lesions in the femoral heads, superficial subchondral BME lesions in the acetabula, deep subchondral BME lesions in the femoral heads, deep subchondral BME lesions in the acetabula, cysts in the femoral heads, and cysts in the acetabula. The lesions designated as cystic were distinguished from the BME lesions by their very distinct outline. Results are tabulated in **Table 2**. In our OA hip cohort, there was no BME lesion in the trochanters or cysts in the acetabula. The only STIR lesions that distinguished OA from HC were superficial subchondral BME lesions in the femoral heads and cysts in the femoral heads. We then searched for similar appearing BME and cystic lesions in AS.

X-ray and MRI in AS Patients

Hip X-rays were systematically evaluated to determine the following characteristics: osteophytes, subchondral sclerosis and femoral head deformity. The upper half of **Table 2** compares X-ray features in HC and AS subjects. Compared with HC subjects, only subchondral sclerosis appeared to be more frequent in AS than that of the control group (22% in AS versus 2% in control, $p = 0.008$). In addition to the above 3 parameters, we also measured joint widths of the involved hips. The mean and standard deviations of joint width in the healthy control group were 4.8 ± 0.74 mm. The range was 3.0 to 6.6 mm. Using the same method of measurement, the mean and standard deviation of the affected AS hips were much lower at 1.5 ± 0.8 mm ($p < 0.00001$). Regression analysis showed that there was a high degree of a statistical relationship between joint width and pain ($r = 0.81$, $p < 0.001$). If the lower limit of normal is set at 3.89 mm, 94.3% of the involved hips in AS were below the normal threshold regardless of the presence or absence of pain. Even more striking, 56.7% of AS patients with no or mild hip pain showed a narrowing of hip-width. We also compared the hip joint width of AS patients who have minimal/no hip pain (3.3 ± 0.66 mm) to mild hip pain (2.15 ± 0.63 mm), to moderate hip pain (1.65 ± 0.26 mm), and severe hip pain (0.93 ± 0.56 mm). The mean hip joint width of each group was significantly different from one another ($p < 0.01$) (**Figure 1**).

In the healthy controls, the percent of hips with BME lesions in trochanters, superficial subchondral BME lesions in the femoral heads, deep subchondral BME lesions in the femoral heads, superficial subchondral BME lesions in the acetabula, deep subchondral BME lesions in the acetabula, cysts in the femoral heads, and cysts in the acetabula were observed in only 0%, 1%, 1%, 0%, 0%, 0% and 0%, of all the hips. The

TABLE 2 | Statistical comparison of X-ray and MRI features of hips in HC, AS and OA subjects.

X ray	Number of individuals	Joint width (mm)	Osteophytes	Subchondral sclerosis	Femoral head deformity			
HC	200	4.8 ± 0.74	1%	2%	0			
AS	300	1.5 ± 0.8*#	2%#	22%*#	1%#			
OA	44	3.2 ± 0.7*#	29%*#	87%*#	32%*#			
MRI	Number of individuals	BME lesions in trochanters	Superficial BME lesions in femoral heads	Superficial BME lesions in acetabula	Deep BME lesions in femoral heads	Deep BME lesions in acetabula	Cysts in femoral heads	Cysts in acetabula
HC	200	0%	1%	1%	0%	0%	0%	0%
AS	300	14%*#	56%*#	38%*#	29%*#	13%*#	0%#	10%*#
OA	44	0%#	30%*#	2%#	4%#	1%#	14%*#	0%#

HC, Healthy control; AS, Ankylosing Spondylitis; OA, Osteoarthritis; BME, bone marrow edema. Numbers in mean ± SD. statistically different from healthy control, p < 0.05~0.0001; # statistically different between AS and OA, p < 0.05~0.0001. All MRI lesions were observed with the STIR sequences.

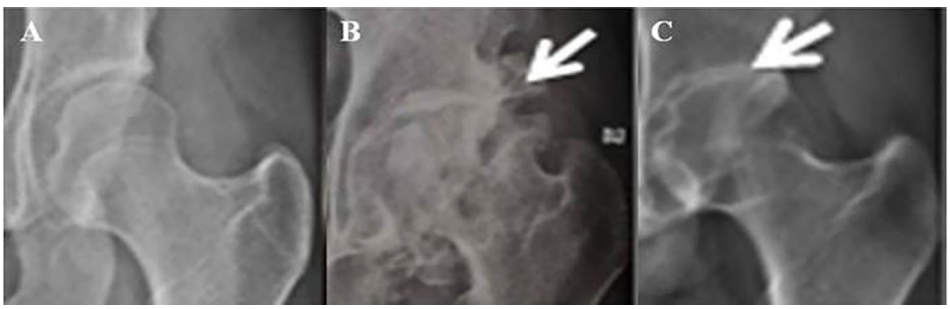

FIGURE 1 | X-rays of hips in a healthy control, OA and AS subject. **(A)** a healthy control subject; **(B)** an OA patient. White arrow points at an osteophyte; **(C)** an AS patients. White arrow points at narrowing of joint space.

corresponding values for AS were 14%, 56%, 38%, 29%, 13%, 0% and 10% respectively (**Table 3**).

All parameters except cysts in the femoral head were statistically more frequent in AS than those in the HC group (p values ranged from < 0.05 to <0.0001). Interestingly, some of the BME lesions appeared to extend inward from the areas where the femoral heads engaged the labrum (**Figure 2**).

When we confined our focus on the group of AS patients with minimal or no pain, we observed three differences from the HC group. Firstly, there were 8% in AS with BME lesions in trochanters. Secondly, 36% of AS showed subchondral BME lesions, and 4% of AS showed cystic lesions. All three MRI lesions were practically non-existent in the HC group. Overall, 42% of those with minimal or no pain in the hips showed one or more MRI lesions. Specifically, we did not observe any fat metaplasia.

X-ray and MRI Changes and Clinical Parameters in the Group With Minimal or No Hip Pain

For X-ray features, we first focus on joint width. The lowest width in the HC group is 3.0 mm. If we consider the lowest 95% Percentile, the lowest limit is 4.0 mm. In the group of patients with minimal/no hip pain, 44% have width less than 3.0 mm and 88% have width less than 4.0 mm. When we examine for MRI changes, 58% show at least one MRI change, 39% show more than one MRI changes. The MRI changes and clinical parameters are shown in the accompanying **Table 4**.

TABLE 3 | Statistical comparison of MRI features in different hip pain subgroups.

AS -hip pain	Number of individuals	BME lesions in trochanters	Superficial BME lesions in femoral heads	Superficial BME lesions in acetabula	Deep BME lesions in femoral heads	Deep BME lesions in acetabula	Cysts in femoral heads	Cysts in acetabula
Minimal/mild [a]	103	8%*	12%*	13%*	5%*	6%*	0%	4%*
Moderate	142	3%	13%	11%	3%	1%	0%	1%
Severe	55	1%	31%#	12%	21%#	6%	0%	5%

*HC, Healthy control; AS, Ankylosing Spondylitis; OA, Osteoarthritis; BME, bone marrow edema. * indicates that the particular minimum/mild hip pain group value is statistically higher than the healthy control group; # represents statistically significant difference between the severe hip pain group and the minimal/mild hip pain; [a]42% of this group has at least one MRI sign. All MRI lesions were observed with the STIR sequences.*

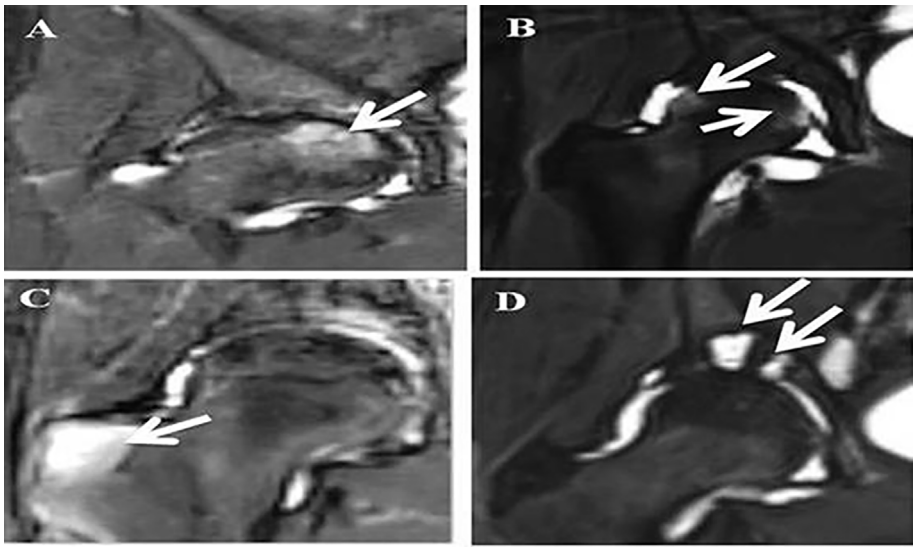

FIGURE 2 | MRI STIR images of hips in AS patients. Legends: **(A)** White arrow points at a deep BME lesion in the femoral head; **(B)** White arrows point at superficial BME lesions in the femoral head; **(C)** White arrow points at a BME lesion in the greater trochanter; **(D)** White arrow points at two cysts in the acetabulum.

TABLE 4 | Comparison of X-ray and MRI changes and clinical parameters in no or minimal hip pain group.

Parameters	Percentage
X-ray width less than 3 mm (%)	44
X-ray width less than 3.85mm (%)	82
X-ray width less than 4 mm (%)	88
at least one MRI change(%)	58
more than one MRI changes(%)	39
MRI features:	
Superficial subchondral BME lesions in the femoral heads (%)	53
Superficial subchondral BME lesions in the acetabula (%)	31
Deep subchondral BME lesions in the acetabula(%)	17
Deep subchondral BME lesions in the femoral heads(%)	14
BME lesions in trochanters(%)	22
Cysts in the acetabula(%)	11
BASDAI ≧4.0(%)	26
High CRP[a](%)	25
High ESR[b](%)	22

BME, bone marrow edema; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; CRP, C-reactive protein (<0.8 mg/dl); ESR, Erythrocyte Sedimentation Rate (Male<15 mm/h, Female<20 mm/h). [a]CRP higher than normal; [b]ESR higher than normal.

DISCUSSION

AS places a huge burden on both patients and society. Inflammation of hip joint can eventually lead to bone damage, which result in decreased physical function and psychological disorders such as anxiety and depression. This further results in the need for help at work, increased sick leave and even incapacity. In addition, patients still need to spend a high amount of medical resources. It is important to note that the burden of disease increases with the course of the disease. Acute inflammatory findings on MRI can predict bone structure changes on late radiographs. Patients with early, typical presentation of AS may not have definite X-ray abnormalities, but further MRI examination may reveal early inflammation. Therefore, early diagnosis and treatment are crucial to improve prognosis.

Hip involvement has been recognized to be present in 19% to 47% of AS patients in several studies (18, 19). The largest of these is a composite of three data sets consisting of more than 2,000 AS patients. In these AS cohorts, hip involvement was present in about a third to a quarter of the patients. As in all studies, hip involvement is associated with a higher degree of functional impairment. Therefore diagnosis, especially early diagnosis should be an important part of AS management. In the above study, hip involvement was defined as either of three parameters: Clinical assessment, radiographic assessment, and need for hip replacement. It is not clear what are the most sensitive methods for either clinical or radiographic assessment. One recent study of 60 AS patients assessed the usefulness of the BASRI-score, which grades the radiographic severity from 0 to 4 using four separate parameters (9). However, the study did not use HC or reveal which particular of the four radiographic parameters was the most useful for identifying early hip involvement.

We know from sacroiliitis that MRI has the potential of showing pathologies before obvious radiographic changes. Two studies are promising in showing that more than 70% of AS patients with clinical hip involvement showed MRI abnormalities (20, 21). The changes they observed were joint effusion, bone marrow edema, and bone erosions. However, there were no HC in their studies. Based on numerous studies on MRI of the sacroiliac joints and the spine, unless MRI of HC for false positives are taken into account, it is impossible to assess which MRI changes of the hips can provide a diagnosis for preclinical and early clinical hip involvements.

We need to examine if patients with minimal or no hip pain might already have MRI changes. To ensure that we could identify MRI lesions, we first used patients with OA as positive

controls. This is because MRI changes have been well described in OA, and typical examples are publically available. We then proceeded to study AS. In MRI images, BME lesions in the STIR sequence were much more frequent in AS (62%) compared to HC (2%) ($p < 0.05$ to 0.0001). Most importantly, 42% of AS with minimal or no hip pain had one or more MRI lesions. This was much more frequent compared to HC ($p < 0.05$). These lesions were observed singly or in combination in the trochanters (8%), femoral heads (12%)and acetabula (13%). For X-ray images, the most significant finding is that there was a high correlation between the joint width of the involved hips with hip pain ($r = 0.81$, $p < 0.001$). Using the HC to set the lower limit of normal, narrowing was observed in 94.3% of the entire AS cohort, and importantly 56.7% of AS patients with no or mild hip pain. To our knowledge, narrowing of hip joint width with minimal or no hip pain has not been reported before.

In AS, overall disease activity in many patients can be controlled by TNF inhibitors, IL-17 inhibitors or Janus Kinase inhibitors. For TNF inhibitors, early treatment is often considered able to retard radiological vertebral progression. However, this has not been studied in hip studies. Part of the difficulty is that there are no markers for early hip disease. The present study offers several promising parameter for future investigation. Since it is not practical to subject every AS patients to MRI of the hips, we studied the X-ray changes in detail. Our results suggest that some AS patients with no or minimal pain might already show joint width narrowing on X-rays. Our results also showed that some of them might have normal acute phase reactants and low disease activity. In clinical practice, it would be reasonable to submit those patients with narrowed joint width to MRI evaluation of their hip joints. This indicated the joint width is a potentially very cost-effective objective imaging parameter to assess for both the presence as well the severity of hip involvement in AS.

The present MRI study adds to the existing information because the cohort is much larger, and because we use healthy control as negative control, and OA as positive control. Further, we categorize patients into groups of symptoms severity.

Limitations

The present study has several shortcomings. The definitions of X-ray and MRI lesions were arbitrarily chosen because there is no standardization available. The method of measuring joint width might vary depending on how the X-rays are taken. In our study, all X-rays were taken with the same protocol. The presence of joint width narrowing in AS is not totally unexpected. What is unexpected is that there is considerable number of patients with joint width narrowing even when they have minimal or no hip pain. Similarly, the presence of MRI changes described is not unexpected. What is unexpected is that they can be present in patients with minimal or no hip pain. Another shortcoming is that we did not detect significant MRI changes with the T1 sequence. This might be partly because we were using a low-resolution machine. Another shortcoming is that the OA we report were used as positive controls. Our OA cohort consisted those with early OA. Any differences between the AS and the OA we observed do not reflect general differences between the two diseases. The most important shortcoming is that this is a cross-sectional study. Any changes we observed might theoretically be transient. However, the study provided parameters which we can use in the future in longitudinal studies.

CONCLUSION

Hip involvement, which occurs in at least 20% of patients with AS, is associated with a high degree of disability. In current clinical practice, accurate diagnosis relies on X-ray, which is usually used only when there is hip pain and probably misses the early cases. This study discovers that AS patients with minimal or no hip pain might already have silent X-ray and MRI changes. Studies are needed to test whether AS patients with X-ray and MRI signs of early hip arthritis should be treated with aggressive medical therapies. The current research provides those X-ray and MRI parameters.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The study and the informed consent have been approved by the ethics committee of Xijing Hospital of Fourth Military Medical University (ID: 20110303-7). The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

QH and PZ contributed to the conception of the work and completed the first draft and final version of the manuscript. QH, ZZ, KZ, JD, and XB contributed to the design of the work. QH, ZZ, KZ, JD, and XB contributed to the data acquisition and analysis. QH, ZZ, KZ, JD, XB, and PZ contributed to interpretation of data. All authors were involved in the manuscript revision and agreed with final approval of the version, and ensured the accuracy of investigation.

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Testing if Micro-CT Is Capable of Quantitating the Extent of Proteoglycan-Aggrecan Induced Axial Spondyloarthritis in Mice

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Objective: Injections of proteoglycan aggrecan (PGA) have been reported to induce axial spondyloarthritis (ax-SpA) in BALB/c mice. It is considered to be a model for radiographic ax-SpA. However, evaluation of the extent of axial disease by histopathological assessment of every intervertebral space is labor-intensive. The objective of our paper is to test the feasibility of Micro Computed Tomography (Micro-CT) in rapidly enumerating the number of intervertebral spaces affected in each mouse.

Methods: Arthritis was induced in BALB/c mice by intraperitoneal injections of PGA. Involvement of several spinal segments, and selected sacroiliac and hip joints were evaluated by histopathology. The involvement of all intervertebral spaces, sacroiliac and hip joints was evaluated by Micro-CT.

Results: BALB/c mice injected with PGA developed histopathology of SpA-like axial lesions, including spondylitis, sacroiliac joint arthritis and hip joint arthritis. Micro-CT allowed us to clearly enumerate the number of lesions in each mouse.

Conclusion: Micro-CT allows quantitative assessment of the extent of axial involvement in PGA-induced mouse spondylitis. This can be a useful tool in assessing therapeutic interventions.

Keywords: spondyloarthritis, proteoglycan, aggrecan, BALB/c, animal models

INTRODUCTION

Axial Spondyloarthritis (ax-SpA) is a chronic inflammatory disease that affects the spine and sacroiliac joints (SIJs). The prototype is Ankylosing Spondylitis (AS) (1). Although the exact pathogenesis of SpA is not known, in AS, Human Leukocyte Antigen (HLA-B27) is a major genetic contributor (2). Tumor necrosis factor (TNF) is one of the main disease-causing cytokines (3, 4). The other known cytokine axis is the interleukin (IL) -23/IL-17 axis (5, 6). Proteoglycan (PG) is a normal component of human and mouse cartilage tissue. Immunizing BALB/c mice with human PG in presence of adjuvants turns it into an autoantigen (7). About 70% of PG-immunised BALB/c mice develop SpA (PG-induced spondylitis, PGISp), with a disease pathology and progression very similar to human AS (8). The success of inhibitors of TNF, IL-17 and Janus Kinase (JAK) in human patients have led to an explosion of clinical therapeutic trials of other modalities. The PGISp mouse

model is potentially a useful screening tool for potential novel therapeutic agents. However, being in the spine, assessment of individual spinal segment by histopathology will be labor-intensive. The purpose of this study was to test the feasibility of Micro-CT as a rapid and accurate tool in quantitative evaluation of the extent of involvement of spine, SIJs and hip joints in this mouse model. The first step of our experiments was to validate the model in our laboratory using histopathology and cytokine assays. The final step was to test if Micro-CT could distinguish each affected intervertebral disc (IVD) and joints from healthy controls (HC).

METHODS

Experimental Animals

Specific pathogen-free (SPF) BALB/c mice purchased from the Experimental Center of the Fourth Military Medical University were placed in a pathogen-free facility. All animal experiments are conducted in accordance with the National Institutes of Health (NIH) guidelines and the guidelines and regulations of the Experimental Animal Research Center at the Fourth Military Medical University.

PGA-Induced SpA Mouse Model

BALB/c female mice (6~8 weeks) were used as experimental animals. Model group (n = 15) was injected with 100g PGA (dissolved in 100uL complete Freund's adjuvant) at time zero. The same dose of PGA (dissolved in 100uL incomplete Freund's adjuvant) was injected intraperitoneally at week 3 and 6 (9). The control group (n = 15) was given intraperitoneal injection of Phosphate Buffer Saline (PBS) and the same doses of adjuvants at the same time schedule as the model group. Mice were sacrificed at week 36 after the third injection. For the care and use of laboratory animals, all procedures used in this study followed the National Institutes of Health guidelines. The institutional Animal Care Committee approved the animal testing program. The Animal Experiment Administration Committee of the Fourth Military Medical University approved all protocols.

Histopathological Examination

The mice were anesthetized and euthanized 36 weeks after the first PGA injection. Tissue samples were fixed in 10% formalin, decalcified in EDTA, and embedded in paraffin. The sections were dewaxed with xylene, dehydrated in a series of graded alcoholic solutions, and then stained with hematoxylin and eosin (H&E).

Micro-CT Imaging

Mice were anesthetized and Micro-CT of the entire skeletons obtained by a Micro-CT machine using a protocol provided by the manufacturer (Inveon; Siemens Healthcare Solutions, Knoxville, Tenn, USA). A companion software reconstructed the images into a 40 m voxel 3D format (Inveon Acquisition Workplace; IAW version 1.5, Siemens Medical Solutions). The effective pixel size (EPS) of the scanning image was 27.8~55.6μm, the scanning time was 30 minute~1 hour, and the average number of scanned slices was 1537.

Cytokine Analysis of Mouse Serum Samples

Blood samples were obtained from isoflurane-anesthetized animals by heart puncture 36 weeks after the third injection. Serum samples were stored at -70°C for batch analysis. A Luminex magnetic beads multiple assay system (R&D Systems, Minneapolis, MN, USA) was used to assay the samples for TNF-α, IL-6, IL-17A, and IL-10, according to manufacturer's instructions.

Statistical Analysis

The results were expressed as the mean ± standard (SD) of the clinical arthritis score. Normally distributed group comparisons are performed using the T-test, non-normally distributed groups using the Man-Whitney U test for. Variance bidirectional analysis (ANOVA) was used to analyze the influence of PGA on clinical scores over time, and then Tukey post-mortem test was performed. All analyses were performed using version 19.0 of SPSS software (SPSS Inc., Chicago, Illinois, USA) and version 7 of GraphPad Prism software (GraphPad Software, San Diego, California, USA).

RESULTS

We did not observe any joint swelling in the peripheral joints. The following results were confined to the spine, the sacroiliac and the hip joints.

Histopathological Features of BALB/c Mice Induced by PGA

Histopathological analysis was performed on mice 36 weeks after the first injection of PGA (n = 10). Control mice were shown in **Figure 1A**. In control mice, the nucleus pulposus was wide and the adjoining bone surfaces were smooth. In PGISp mice, we observed extensive narrowing of the intervertebral space (**Figures 1B, C**), and in certain areas the intervertebral space was bridged by bone (**Figure 1D**). When observed at ten times higher magnification, many chondrocytes were observed next to the much narrowed intervertebral space (**Figure 2**).

Micro-CT Results

Figure 3A shows several control vertebral bodies with clearly defined intervertebral space. **Figures 3B, C** show the control SIJs and a hip joint respectively. The joint surfaces appeared smooth, and the joint space distinct. In PGISp mice group, the intervertebral space became much narrower (**Figure 3D**). The surfaces of the SIJs appeared irregular (**Figure 3E**). The space of the hip joints also became much narrower (**Figure 3F**).

Serum Cytokine Analysis

Serum samples (n=9) were obtained at week 36 and cytokine levels were measured. The expressions of IL-6, IL-17A and TNF-α were significantly higher in the PGA-induced mice compared with those

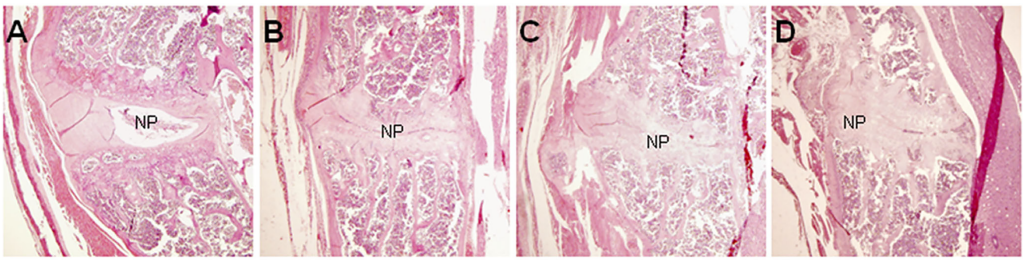

FIGURE 1 | Pathological changes of spondylitis in BALB/c mice induced by PGA injections (HE, × 40). At the center of each figure is the IVD. **(A)** HC mouse; **(B)** Extensive narrowing of intervertebral space in a PGISp mouse; **(C)** More extensive narrowing of intervertebral space; **(D)** Bridging bone formation in part of the intervertebral space; NP= nucleus pulposus.

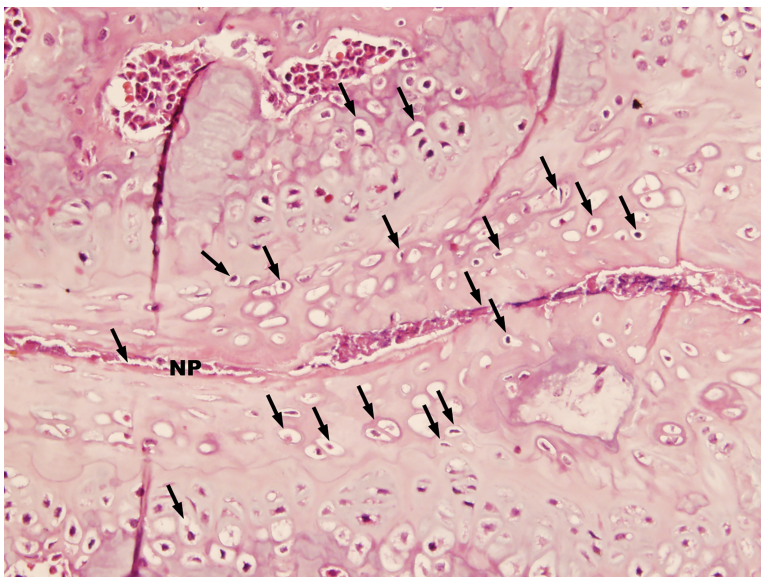

FIGURE 2 | Advanced pathological changes of an intervertebral space in a PGISp mouse (HE, × 400). A large number of chondrocytes (black arrows) appeared next to the very much narrowed intervertebral space. Part of the intervertebral space seemed to have disappeared.

in the control group (**Figures 4A–C**). Compared with the control group, the expression of IL-10 in mice that induced by PGA was significantly decreased (**Figure 4D**).

Evaluation of Number of Affected Intervertebral Space and Affected SIJs and Hip Joints as Visualized by Micro-CT

All the intervertebral spaces, the sacroiliac and hip joints appeared normal in the control mice. In **Table 1**, we designate the observation of each intervertebral space of each PGA-induced model mouse as positive or negative. A positive sign indicates an abnormal appearing space/joint. A negative sign indicates a normal space/sign. Data of sacroiliac and hip joints were not available in 3 mice because we only had X-ray images of those joints in those mice. The resolution in X-ray images were too low to be interpreted.

All PGISp mice showed changes in some intervertebral spaces. Pathology mainly concentrated in T6∼T12, L1∼L2, L3∼L4, S1∼S2. Changes in SIJs and hip joints were observed in 100% and 57% of PGA-induced model mice (**Table 1**).

DISCUSSION

The IVD is a complex fibrochondrocyte structure containing chondrocytes and fibroblast-like cells embed in a relatively vascular environment. The extracellular matrix of IVD is mainly composed of collagen and PG, the nucleus pulposus of type II collagen and aggregative PG, and the anulus fibrosus of type I collagen and aggregative PG. PG is also present in articular joints. In theory, an autoimmune response towards PG will lead to inflammatory diseases in the intervertebral discs as well the

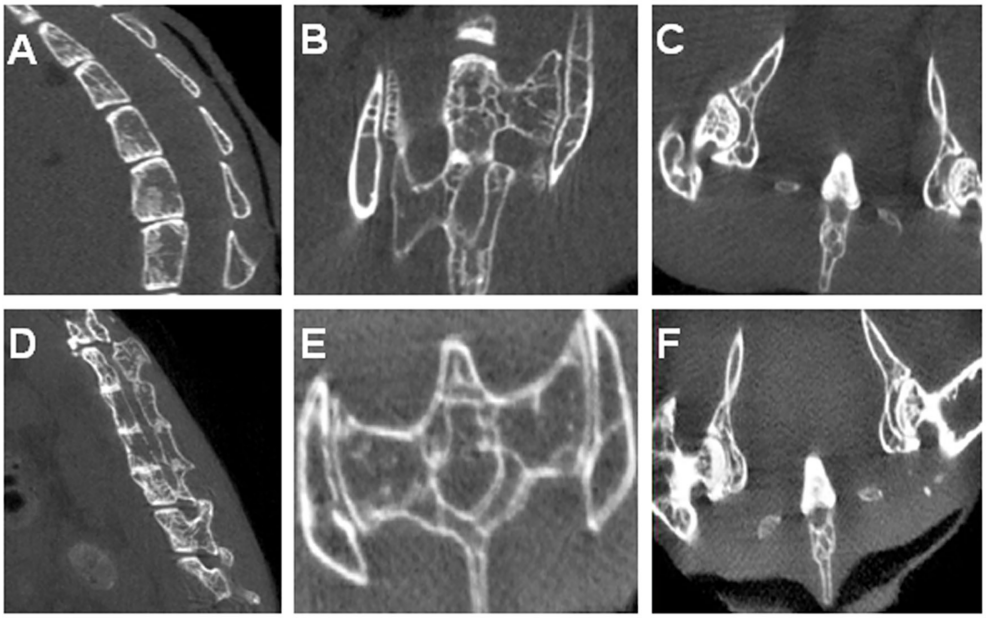

FIGURE 3 | Micro-CT results: **(A)** A control mouse showing physiological spinal curvature, intact bone, smooth articular surface, and substantial intervertebral space; **(B)** SIJs of a control mouse; **(C)** Hip joints of a control mouse; **(D)** Spine of an PGA-induced model mouse showing loss in curvature, irregular and sclerotic articular surfaces, and narrowing of intervertebral discs. Some of the vertebral bodies appeared fused; **(E)** SIJs of an PGA-induced model mouse showing irregular joint surfaces; **(F)** Hip joints of an PGA-induced model mouse showing irregular joint surfaces and narrowing of joint spaces.

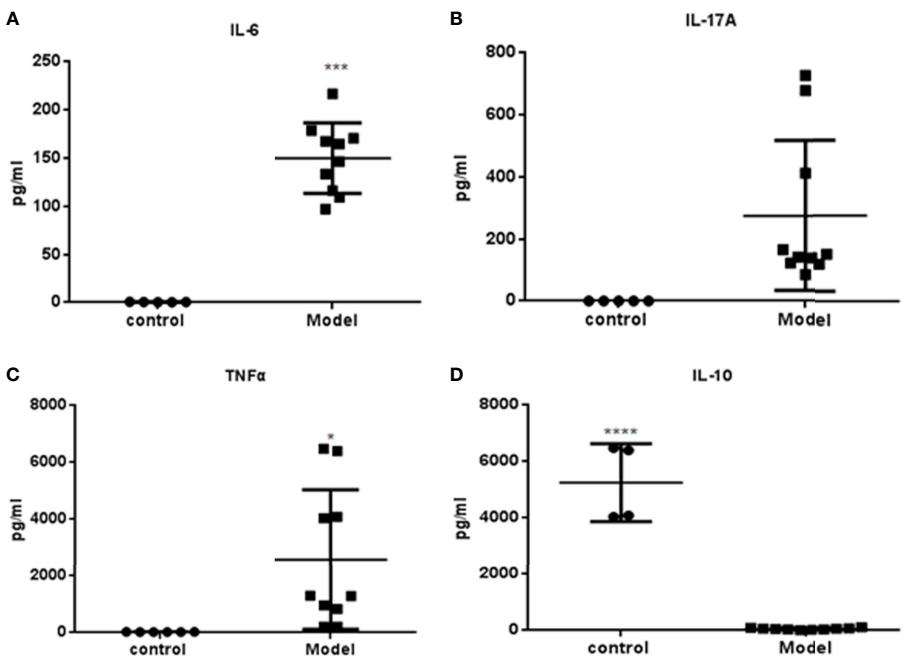

FIGURE 4 | Cytokines measured by Luminex determination (R&D Systems, USA): Serum was obtained from BALB/c mice immunized with PGA or PBS at week 36. **(A)** IL-6; **(B)** IL-17A; **(C)** TNF-α; **(D)** IL-10. The expressions of IL-6, IL-17A and TNFα were higher in the PGA-induced model group compared with those in the control group. The expression of IL-10 in the PGA-induced model group was lower than that in the control group (control group = 4, PGA-induced model group = 9). *$p < 0.05$; ***$p < 0.01$; ****$p < 0.001$, compared with control group.

TABLE 1 | X-ray/Micro-CT assessment of individual intervertebral space as well as sacroiliac and hip joints in PGISp mice.

X-ray	IVD Inflammation	M1	M2	M 3	M 4	M 5	M 6	M 7	M8	M 9	M10	M 11	M12	M 13	M 14	Numbers of Positive mice
	C1-C2	−	−	−	−	−	−	−	−	+	+	+	+	+	+	6
	C3-C4	−	−	−	−	−	−	−	−	+	+	+	+	+	+	6
	C4-C5	−	−	−	−	−	−	−	−	+	+	+	+	+	+	6
	C5-C6	+	+	−	−	−	−	−	−	−	−	−	+	+	+	5
	C6-C7	+	+	−	+	−	−	−	−	−	−	−	+	+	+	6
	T1-T2	+	+	−	−	−	−	−	−	−	−	−	−	−	−	2
	T2-T3	+	+	+	+	+	+	+	−	−	−	−	−	−	−	7
	T3-T4	+	+	+	+	+	+	+	−	−	−	+	−	−	−	8
	T4-T5	+	+	−	−	−	−	−	−	−	−	−	+	+	−	4
	T5-T6	+	+	−	−	−	−	−	−	−	−	−	+	+	−	4
	T6-T7	+	+	+	+	−	+	+	−	+	+	+	+	+	+	12
	T7-T8	+	+	+	+	−	+	+	−	+	+	+	+	+	+	12
	T8-T9	+	+	+	+	−	+	+	−	+	+	+	+	+	+	12
	T9-T10	+	+	+	+	−	+	+	−	+	+	+	+	+	+	12
	T10-T11	+	+	+	+	−	+	+	−	+	+	+	+	+	+	12
	T11-T12	+	+	+	−	+	−	+	−	+	−	+	−	+	−	8
	L1-L2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	14
	L2-L3	+	+	+	−	+	−	+	+	+	−	+	−	+	−	9
	L3-L4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	14
	S1-S2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	14
	S2-S3	+	+	+	+	+	+	+	+	+	+	+	−	−	−	11
	S3-S4	+	+	+	+	−	+	+	+	+	+	+	+	−	−	12
Micro-CT	R-SIJ	+	+	+	−	NA	NA	NA	+	+	+	+	+	−	−	7
	L-SIJ	+	+	+	+	NA	NA	NA	+	+	+	+	+	−	−	8
	R-hip	+	+	+	+	NA	NA	NA	+	+	+	−	−	−	−	7
	L-hip	+	+	−	−	NA	NA	NA	−	+	+	−	−	−	−	4
	Shoulders	−	−	−	−	NA	NA	NA	−	−	−	−	−	−	−	0
	Ankles	−	−	−	−	NA	NA	NA	−	−	−	−	−	−	−	0

M, PGISp mouse; IVD, intervertebral disc; C, cervical spine; T, thoracic spine; L, lumbar spine; S, sacral vertebrae; R-SIJ, right sacroiliac joint; L-SIJ, left sacroiliac joint; R-hip, right-hip joint; L-hip, left-hip joint; NA, data not available.

peripheral joints. Indeed, PG induced arthritis is a well-established mouse model (10). It is different from collagen-induced mouse arthritis. In collagen-induced arthritis, the highest incidence is in DBA/1 mice. In PG induced arthritis, the highest incidence is in the BALB/c mice. The incidence of arthritis in the progeny of BALB/c mice hybridization with DBA/2 mice was only 43.5%, suggesting that the arthritis was related to the Major Histocompatibility Complex (MHC) gene, although the specific susceptibility gene loci of BALB/c mice have not yet been determined (11, 12).

In all reports of PG induced arthritis, both peripheral and axial arthritis are present. However, even in BALB/c mice the phenotype of the arthritis varies among subclones of mice in the same institute (13). The particular colony we immunized did not show any peripheral arthritis, but showed disease in the intervertebral disc, the hip and the SIJs. Arthritis mice also showed higher levels of TNF-α and IL-17A in the sera. These features are similar to patients with AS. Although the arthritis in AS is not caused by an autoimmune response against PG, there is probably some similarity in the downstream processes. Hence, the mouse model can be used for screening of potential therapeutic agents. What is needed is an accurate and convenient tool to quantitate the extent of disease. Being in the spine, disease involvement is much more difficult to observe compared to the peripheral joints. With at least twenty IVDs, it will be labor-intensive to assess each by histopathology. Our results with Micro-CT demonstrate that it provides clear images of the IVD, the sacroiliac and the hip joints.

A major limitation of this paper is that it does not address the pathogenesis of PGA arthritis. The serum cytokine tests and the immunohistology were carried out to ensure it was an inflammatory disease with tissue destruction and changes in cytokines parallel to those with human SpA. The only conclusion which we can be confident are observations from the micro-CT.

CONCLUSION

Micro-CT is an accurate and convenient tool to quantitate disease involvement in PGA-induced mouse arthritis in the spine, sacroiliac and hip joints.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Fourth Military Medical University.

AUTHOR CONTRIBUTIONS

QH, ZZ, QL, and PZ contributed to the conception of the work, completed the first draft, and final version of the manuscript. QH, ZZ, QL, KZ, and PZ contributed to the design of the work. QH, ZZ, QL, KZ, FY, XF, XL, JD, WZ, and RX contributed to the data acquisition and analysis. QH, ZZ, QL, and PZ contributed to interpretation of data. All authors were involved in the manuscript revision and agreed with final approval of the version, and ensured the accuracy of investigation. All authors contributed to the article and approved the submitted version.

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frontiers in Immunology

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Imbalance of Peripheral Lymphocyte Subsets in Patients With Ankylosing Spondylitis: A Meta-Analysis

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Ankylosing spondylitis is a complicated consequence of genetic predisposition and environmental factors. Enthesitis is believed to be the hallmark of ankylosing spondylitis, and the chronic inflammatory state of this disease is perpetuated by the disturbances of both the innate immune system and the acquired immune system. To clarify the alteration of immune system in patients with AS, we conducted a meta-analysis concerning the proportions of major lymphocyte subsets in the peripheral blood of AS patients. We systematically searched PubMed and China National Knowledge Infrastructure (CNKI) for articles related to this subject. A total of 95 articles involving 4,020 AS patients and 3,065 healthy controls were included in the analysis. This meta-analysis is performed on R platform using R package "meta", and Egger's tests were used to determine the presence of publication bias. Results showed that the percentages of T cells, NK cells and NKT cells were not significantly different between AS patients and healthy controls, but B cells were significantly increased. Among the subsets of T cells, the proportions of CD4+ T cells, Th17 cells, Tfh cells as well as Th1/Th2 ratio were significantly increased, while Tregs were significantly decreased. Subgroup analysis showed that the proportions of Th17 among both PBMCs, T cells and CD4+ T cells were significantly elevated, while Tregs were only significantly lower in PBMCs. Subgroup analysis also demonstrated that Tregs defined by "CD4+CD25+FoxP3+", "CD4+CD25+CD127low"or "CD4+CD25+CD127-"were significantly downregulated, indicating that the selection of markers could be critical. Further study is warranted in order to elucidate the complicated interactions between different lymphocyte subsets in AS patients. This study implied that the disequilibrium between Th17 and Tregs, as well as between Th1 and Th2 could contribute to the pathogenesis of ankylosing spondylitis, further cementing the understanding that ankylosing spondylitis is a consequence of disrupted balance of innate immune system and acquired immune system.

Keywords: Ankylosing spondylitis, lymphocyte, immune system, flow cytometry, Th17 & Treg cells

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INTRODUCTION

Ankylosing spondylitis belong to the group of diseases known as spondyloarthrpathies, which is a spectrum of diseases encompassing psoriatic arthritis, reactive arthritis and undifferentiated spondyloarthritis (1). Clinical manifestations of ankylosing spondylitis include articular manifestations and extra-articular manifestions. The articular manifestations mainly involve axial skeleton presenting as inflammatory back pain, with peripheral oligoarthritis present in some of the patients, while the extra-articular manifestations include uveitis, gut inflammation and dactylitis (2–4). To date, the pathogenesis of ankylosing spondylitis has not yet been fully elucidated. Previous studies have revealed that ankylosing spondylitis is a consequence of genetic background and environmental factors, with HLA-B27 stepping into the limelight of research upon the discovery that HLA-B27 can be present in as many as 90% of patients with AS (5).

How HLA-B27 causes the disease of ankylosing spondylitis remains unclear, though several hypotheses have been put forward attempting to connect the dots (6, 7). Yet, it is undisputed that the disturbances of the immune system eventually perpetuate this disease (8–10). Unlike autoimmune diseases like systemic lupus erythematosus and rheumatoid arthritis, it is not the autoreactive B cells secreting auto-antibodies that should be held accountable, since no antibody is widely acknowledged to be detected in patients with ankylosing spondylitis (11). Instead, such disturbances in the immune system in patients with AS are the result of complicated interactions between the innate immune system and the adaptive immune systems (10). The successful application of biologics, especially TNF-α inhibitors, provide substantial evidence that by blocking cytokines characteristic of the innate immune system, the inflammatory status can be greatly alleviated (12, 13). On the other hand, numerous studies have added to the confirmation of the fact that AS is driven by the imbalances of lymphocyte subsets, especially the Th17/Tregs and Th1/Th2 imbalances, disrupting the equilibrium of the immune system (14, 15). The specific CD4+ T cell subset of Tregs possess immunosuppressive features (16), and the incapability of Tregs may allow the over-secretion of pro-inflammatory cytokines, especially IL-17, which is a potent pro-inflammatory cytokine secreted by Th17 and plays an important role in mediating bone damage (17). Meanwhile, the hyperactivation of the Th1 effector T cell lineage may secrete abundant IFNγ and TNF-α (18), leading to the chronic inflammatory state of the disease.

However, different studies have provided conflicting data regarding the direction and extent of the imbalance of lymphocytes. Most studies suggested that the percentages of Tregs were significantly decreased in patients with AS, yet a few studies found that Tregs might be increased in the peripheral blood of AS patients, arguing that the increase of Tregs might be the result of an attempt to enhance immune tolerance to control the immune response. More intriguingly, the proportions of NK cells is the peripheral blood of AS patients were heavily debated. It has been hypothesized that KIR3DL2, an inhibitory receptor expressed on NK cells, might inhibit apoptosis of NK cells once ligated with HLA-B27, leading to an excess of NK cells in the peripheral blood. In the meanwhile, a few studies observed a significant decrease in the proportions of NK cells in AS patients. Based on previous studies, we hypothesized that the elevation of Th17 and the downregulation of Tregs were pivotal in the pathogenesis of AS, while the th1/th2 polarization might also be involved. In order to clarify the actual proportions of different subsets of lymphocytes, we conducted a meta-analysis concerning the lymphocyte imbalances in the peripheral blood in patients with AS, with healthy donors as the control.

METHODS

Data Sources and Searches

We searched the relevant studies using PubMed, Cochrane, Medline and China National Knowledge Infrastructure (CNKI). The literature search strategy used the following terms: ("ankylosing spondylitis") AND ("lymphocyte subsets" OR "T cell" OR "B cell" OR "Th1" OR "Th2" OR "Th17" OR "Treg" OR "NK cell" OR "NKT cell" OR "gamma delta T cell" OR "flow cytometry"). The publication date was set before April 1, 2021, and all potential eligible studies were screened except for animal experiments or reviews. Some of the studies listed in the reference were retrieved through reference literature in related articles.

Study Selection

The inclusion criteria were as follows: (a) original research; (b) human research; (c) studies with full text available; (d)studies that provided data concerning proportions of certain lymphocyte subsets in peripheral blood of AS patients; (e) studies that provided information concerning flow cytometry experiment protocol and subject characteristics.

The following criteria is used to exclude studies from the final analysis: (a) Studies that did not provide data in the form of mean and standard deviation, or data that could not be transformed; (b) Studies focusing on certain tissue instead of peripheral blood; (c)Duplicates already included once in the analysis.

Two independent researchers (Dong Liu and Budian Liu) extracted data from eligible articles according to the inclusion criteria, while a third investigator settled any disagreements (Churong Lin). Extracted data included author's name, publication year, baseline characteristics, number of patients and healthy controls, markers of lymphocytes, diagnostic criteria and proportions of each lymphocyte subset in PBMC or T cells or CD4+ T cells. Data were recorded as mean and standard deviation. If the percentages of lymphocytes were presented as median or interquartile range yet no obvious skewing is identified, the data is transformed to mean and standard deviation. (SD = IQR/1.35) The Newcastle-Ottawa Quality Assessment Scale was used to assess the quality of studies.

Statistical Analysis

This meta-analysis was perfomed on the R platform, using R package "meta" [v4.13-0; (19)]. The Cochrane chi-squared

test was used to assess the heterogeneity of the included studies. If the heterogeneity of the studies were high (), then the random-effects model was employed to conduct the analysis. Subgroup analysis was performed when it was deemed necessary to break down the analysis on levels of comparison (PBMC, T cells or CD4+ T cells) or based on different markers. Considering the heterogeneity of the literature since different classification criteria were applied, and disease activity of the patients varied across studies, we also conducted subgroup analysis based on classification criteria and disease activity. Publication bias was assessed by the Egger's test (p≥0:05). Sensitivity analyses was conducted to test the robustness of the results.

RESULTS

Study Characteristics

Based on the methods stated above, a total of 2,982 articles were retrieved. We excluded 700 articles since they were duplicates. Next, through screening the abstracts of the articles, a total of 384 articles were included. After carefully examining the articles, articles without full text or failing to provide original data were excluded, leaving 95 articles eligible to be included in the final meta-analysis. Flow chart of the literature search process can be seen in **Figure 1**.

This meta-analysis included 4020 AS patients and 3065 healthy controls from 95 eligible studies. The features of these studies can be seen in **Table 1**. Of all the studies, 19, 19, 13 and 3 studies provided data on the proportion of T cells, B cells, NK cells and NKT cells. As for the subsets of T cells, 37, 32 and 3 studies focused on CD4+ T cells, CD8+ T cells and γδ T cells. Delving into the CD4+ T cells, 19, 12, 28, 46 and 3 studies presented data on the proportions of Th1, Th2, Th17, Tregs and Tfh cells. Six and 7 studies further discussed the Th1/Th2 proportions and Th17/Treg proportions. All studies had a NOS score of 3-7; the qualities of these studies were moderate. The original data can be seen in **Supplemental Material 1**.

Proportions of T Cells

Firstly, we conducted a meta-analysis on the proportions of T cells in PBMC between AS patients and healthy controls, as well as the subsets of T cells in the corresponding category (**Figure 2**). Results showed that there is no significant difference in the T cell

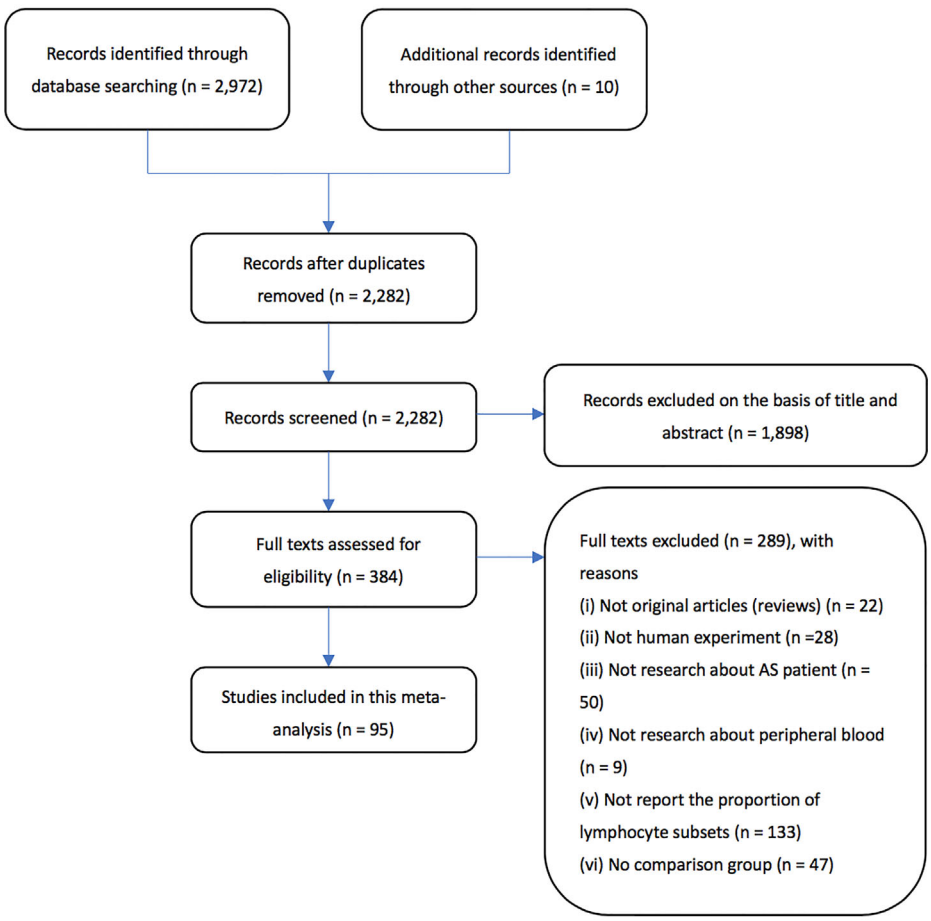

FIGURE 1 | Flow chart of the literature search and study selection process.

TABLE 1 | Characteristics of 95 studies included in this meta-analysis.

Author (Ref.)	Publish year	Country	Case numbers (AS/HC)	Lymphocyte subsets discussed	Age (year) AS/HC	Disease activity	Diagnosis criteria	NOS score	Database
An et al. (20)	2019	China	73/85	Th17/Treg ratio	nr	nr	mNY1984	5	Medline; PubMed
Appel et al. (21)	2011	Germany	19/20	Treg	40.9 ± 13.8/nr	nr	mNY1984	4	Medline; PubMed
Bautista et al. (22)	2014	Norway	25/50	Tfh	56 ± 14.8/nr	nr	mNY1984	5	Medline; PubMed
Bidad et al. (23)	2013	Iran	18/18	Th17; Treg	34 ± 2/33 ± 1	BASDAI≥4	mNY1984	6	Medline; PubMed
Brand et al. (24)	1997	Germany	21/29	B; CD4+T; CD8+T	42 ± 14/47 ± 16	nr	mNY1984	5	Medline; PubMed
Cai and Xiao (25)	2013	China	40/20	Treg	29 ± 9.4/28.4 ± 10.3	nr	mNY1984	7	CNKI
Cai et al. (26)	2005	China	30/20	B; T; CD4+T; CD8+T	nr	nr	mNY1984	4	CNKI
Cao et al. (27)	2004	Sweden	10/29	Treg	nr	nr	mNY1984	4	PubMed
Chen et al. (28)	2013	China	61/36	Th1; Th17; Treg	25 ± 8.2/25 ± 7	nr	mNY1984	7	CNKI
Chen et al. (29)	2011	Taiwan (China)	23/25	B; T; CD4+T; Treg	nr	nr	mNY1984	6	Medline; PubMed
Cheng (30)	2007	China	25/21	CD4+T	28 ± 9/27 ± 6	nr	mNY1984	6	CNKI
Dejaco et al. (31)	2010	Austria	22/17	Treg	40.9 ± 12.7/ 40.3 ± 23.4	nr	nr	3	Medline; PubMed
Deng et al. (32)	2019	China	49/100	Th1; Th2; Th1/Th2 ratio	28.31 ± 6.72/ 27.38 ± 6.39	nr	mNY1984	7	CNKI
Deng et al. (33)	2018	China	91/50	T; CD4+T; CD8+T	nr	nr	mNY1984	6	CNKI
Dong et al. (34)	2006	China	30/30	T; CD4+T; CD8+T	nr	nr	mNY1984	5	CNKI
Duan et al. (35)	2017	China	21/16	T; CD4+T; CD8+T; Treg	37 ± 9.8/34.6 ± 10.1	BASDAI≥4	mNY1984	6	Medline; PubMed
Dulic et al. (14)	2017	Hungary	7/10	CD4+T; CD8+T; Th1; Th2; Th1/Th2 ratio; Th17; Treg; Th17/Treg ratio	nr	nr	mNY1984	6	Medline; PubMed
Fattahi et al. (36)	2018	Iran	30/15	Th17; Treg	31.4 ± 9.1/ 32.1 ± 8.2	BASDAI≥4	mNY1984	6	Medline; PubMed
Forger et al. (37)	2009	Switzerland	15/18	Treg	nr	nr	mNY1984	5	PubMed
Gao et al. (38)	2012	China	40/37	Th17; Treg	29.1 ± 8.6/ 26.7 ± 6.9	nr	mNY1984	6	CNKI
Guo et al. (39)	2012	China	98/76	CD4+T; CD8+T	nr	nr	nr	5	CNKI
Hajialilo et al. (40)	2019	Iran	24/35	Th17	nr	BASDAI≥4	ASAS2009	6	Cochrane; Medline; PubMed
Han et al. (41)	2006	China	69/50	B; T; CD4+T; CD8+T	nr	nr	mNY1984	5	CNKI
He et al. (42)	2012	China	32/50	B; CD4+T; NK	nr	nr	nr	4	CNKI
Hu et al. (43)	2019	China	60/40	B; CD4+T; CD8+T; NK	nr	nr	mNY1984	5	CNKI
Hu et al. (44)	2013	China	32/30	T; CD4+T; CD8+T; Th1; Th2	34 ± 3.89/36 ± 3.76	nr	mNY1984	4	CNKI
Huang et al. (45)	2009	China	20/9	CD4+T; Treg	nr	nr	mNY1984	4	CNKI
Huang et al. (46)	1990	China	9/9	CD4+T; CD8+T	nr	nr	mNY1984	4	CNKI
Ji et al. (47)	2014	China	20/20	Treg	nr	nr	mNY1984	7	Medline; PubMed
Kenna et al. (48)	2012	Australia	17/20	γδT; Th17	39.47 ± 13.6/ nr	nr	mNY1984	6	Medline; PubMed
Kim et al. (49)	2019	South Korea	49/53	CD4+T; CD8+T; NK	36.4 ± 10.8/ 34.9 ± 9	nr	mNY1984	6	Medline; PubMed
Klasen et al. (50)	2019	Germany	14/5	Th17	42.7 ± 3.15/nr	nr	mNY1984	5	Medline; PubMed
Li (51)	2019	China	64/60	Th17; Treg	33.26 ± 5.74/ 35.84 ± 6.19	nr	mNY1984	7	CNKI

(Continued)

TABLE 1 | Continued

Author (Ref.)	Publish year	Country	Case numbers (AS/HC)	Lymphocyte subsets discussed	Age (year) AS/HC	Disease activity	Diagnosis criteria	NOS score	Database
Li et al. (52)	2013	China	222/68	Th17; Treg	33.6 ± 8/34.1 ± 10.6	BASDAI≥4	mNY1984	6	Medline; PubMed
Li et al. (53)	2009	China	30/10	Th1; Th2; Th1/Th2 ratio	nr	BASDAI≥4	mNY1984	5	CNKI
Li et al. (54)	2008	China	50/21	T; CD8+T	25 ± 8/25 ± 5	nr	mNY1984	6	CNKI
Liao et al. (55)	2015	Taiwan (China)	69/30	Treg	39.6 ± 12.7/ 44.3 ± 10.5	nr	mNY1984	7	Medline; PubMed
Limon-Camacho et al. (56)	2012	Mexico	39/25	Th1; Th2; Th17; Treg	32 ± 13/32 ± 8	BASDAI≥4	mNY1984	4	PubMed
Lin et al. (57)	2008	China	66/30	CD4+T; CD8+T	29.7 ± 9.6/ 26.7 ± 6.7	nr	mNY1984	6	CNKI
Lin et al. (58)	2009	China	66/30	B	29.7 ± 9.6/ 26.7 ± 6.7	nr	mNY1984	6	Cochrane; Medline; PubMed
Liu and Feng et al. (59)	2017	China	38/38	Th1; Th17	39.3 ± 3.4/ 40.4 ± 3.9	nr	mNY1984	6	CNKI
Liu et al. (60)	2016	China	60/20	Treg	35 ± 10.7/41.9 ± 11.7	nr	mNY1984	5	CNKI
Liu et al. (61)	2012	China	60/30	Treg	31.5 ± 9.1/nr	nr	mNY1984	6	CNKI
Liu et al. (62)	2010	China	30/20	Th1/Th2	26 ± 3.69/ 25.15 ± 3.79	nr	mNY1984	6	CNKI
Long et al. (63)	2018	China	65/20	CD4+T; Tfh	27.8 ± 8.5/ 31.4 ± 7.4	nr	mNY1984	6	Medline; PubMed
Ma et al. (64)	2011	China	36/32	B; CD4+T; CD8+T; NK	23.1 ± 4.8/ 25.8 ± 3.6	nr	mNY1984	5	CNKI
Ma et al. (65)	2011	China	43/20	B; T; CD4+T; CD8+T; NK	nr	nr	mNY1984	4	CNKI
Ma et al. (66)	2004	China	25/30	B; T; CD4+T; CD8+T; NK	nr	nr	mNY1984	5	CNKI
Meng et al. (67)	2015	China	42/20	CD8+T;	32.4 ± 9.3/ 29.5 ± 8.4	nr	mNY1984	6	CNKI
Mo et al. (68)	2019	China	30/23	B; CD4+T; CD8+T; γδT; NK	40.7 ± 3.18/ 45.71 ± 2.6	nr	mNY1984	7	CNKI
Pishgahi et al. (69)	2020	Iran	31/35	Th17; Treg	nr/41.89 ± 11.29	nr	ASAS2009	5	Medline; PubMed
Shan et al. (70)	2015	China	20/10	Treg	nr	BASDAI≥4	mNY1984	6	Medline; PubMed
Shen et al. (71)	2009	China	10/16	Th17	46.05 ± 11.51/ nr	nr	mNY1984	3	Medline; PubMed
Suen et al. (72)	2008	Taiwan (China)	23/26	Treg	43 ± 12/37 ± 12	nr	nr	4	Medline; PubMed
Szalay et al. (15)	2012	Hungary	13/9	CD4+T; CD8+T; Th1; Th2; Th1/Th2 ratio; Th17/Treg ratio	43.7 ± 9.2/nr	BASDAI≥4	mNY1984	4	Medline; PubMed
Szanto et al. (73)	2008	Hungary	42/52	B; T; CD4+T; CD8+T; Th1; Th2; NK	nr	BASDAI≥4	mNY1984	5	Medline; PubMed
Thoen et al. (74)	1987	Norway	31/15	CD4+T; CD8+T	32 ± 1.8/nr	nr	mNY1984	3	Medline; PubMed
Toussirot et al. (75)	2009	France	32/15	Treg	42.9 ± 1.1/ 44.4 ± 0.8	nr	nr	4	PubMed
Wang et al. (76)	2020	China	90/90	Th17; Treg; Th17/Treg ratio	43.27 ± 8.19/ 43.55 ± 8.6	nr	nr	5	CNKI
Wang et al. (77)	2018	China	30/30	Th1; Th17; Treg	31.2 ± 4.1/nr	nr	mNY1984	6	CNKI
Wang et al. (78)	2018	China	26/26	Treg	33.5 ± 8.4/ 31.5 ± 10.2	ASDAS ≥ 2.1	mNY1984	4	Medline; PubMed
Wang et al. (79)	2016	China	50/50	CD4+T; γδT	28.53 ± 8.15/ 27.93 ± 8.52	nr	mNY1984	7	Medline; PubMed
Wang et al. (80)	2015	China	78/30	Treg; Th17/Treg ratio	26 ± 7.8/25 ± 8	nr	mNY1984	6	CNKI
Wang et al. (81)	2015	China	45/20	T; CD8+T; Th17; Treg	nr/55.05 ± 6.42	nr	nr	5	Medline; PubMed
Wang et al. (82)	2012	China	60/44	B; T; CD4+T; CD8+T; NK	nr	nr	mNY1984	6	CNKI

(Continued)

TABLE 1 | Continued

Author (Ref.)	Publish year	Country	Case numbers (AS/HC)	Lymphocyte subsets discussed	Age (year) AS/HC	Disease activity	Diagnosis criteria	NOS score	Database
Wang et al. (83)	2008	China	30/20	CD4+T; CD8+T; Th1; Th2	26 ± 3.69/ 25.15 ± 3.79	nr	mNY1984	6	CNKI
Wei et al. (84)	2017	China	131/127	B; T; CD4+T; CD8+T; Treg; NK	27 ± 8/26 ± 9	nr	mNY1984	6	CNKI
Wu (85)	2014	China	60/60	Tfh	26.9 ± 7.8/ 24.3 ± 5.6	nr	mNY1984	6	CNKI
Wu et al. (86)	2011	China	51/49	Treg	nr	BASDAI≥4	mNY1984	6	Medline; PubMed
Wu et al. (87)	2011	China	24/30	B	35 ± 14/33 ± 12	nr	mNY1984	6	CNKI
Xu et al. (88)	2019	China	18/9	Th17; Treg	39.4 ± 2.3/ 42.6 ± 4.3	nr	mNY1984	6	Medline; PubMed
Xu et al. (89)	2018	China	69/22	CD4+T; CD8+T; NKT	nr	nr	mNY1984	6	CNKI
Xu (90)	2013	China	24/22	Th1; Th17; Treg	24.3 ± 8.5/ 27.9 ± 8.6	nr	mNY1984	6	CNKI
Xu et al. (91)	2011	China	78/50	B; T; CD4+T; CD8+T; Th1; Th2	nr	nr	nr	5	CNKI
Xue et al. (92)	2015	China	38/30	Th17; Treg	29.93 ± 9.82/ 30.58 ± 8.39	nr	mNY1984	6	CNKI
Xue et al. (93)	2008	China	89/42	T; CD4+T; CD8+T	nr	nr	nr	5	CNKI
Yang et al. (94)	2020	China	67/50	B; Th1; Th2; Th17	nr	ASDAS ≥ 1.3	mNY1984	6	Medline; PubMed
Yang et al. (95)	2018	China	30/30	T; NKT	29.3 ± 5.9/ 31.1 ± 6.7	nr	mNY1984	6	CNKI
Yang et al. (96)	2017	China	40/40	Treg	32.53 ± 9.76/ 33.7 ± 10.06	nr	mNY1984	6	CNKI
Yang et al. (97)	2016	China	38/31	Treg	28.9 ± 10.8/ 29.1 ± 8.1	nr	mNY1984	7	CNKI
Yang et al. (98)	2007	China	60/30	B; T; CD4+T; CD8+T	nr	nr	mNY1984	6	CNKI
Ye et al. (99)	2013	China	21/27	Treg	36.6 ± 10.2/ 37.9 ± 9.1	nr	mNY1984	3	Medline; PubMed
Zhang et al. (100)	2019	China	60/30	Th17; Treg; Th17/Treg ratio	43 ± 11/32 ± 12	nr	mNY1984	5	CNKI
Zhang et al. (101)	2019	China	39/41	B; T; CD4+T; CD8+T; NK	28.87 ± 8.31/ 27.05 ± 6.63	nr	mNY1984	6	CNKI
Zhang et al. (102)	2014	China	60/60	Th1; Th17; Treg	39 ± 3.2/39.2 ± 3.1	nr	mNY1984	6	CNKI
Zhang et al. (103)	2014	China	10/10	Th17	nr	nr	mNY1984	4	CNKI
Zhang et al. (104)	2012	China	32/20	Th1; Th17	36.6 ± 10.2/ 37.9 ± 9.1	nr	mNY1984	6	Medline; PubMed
Zhang et al. (105)	2008	China	78/50	Treg; NK	26.1 ± 6.8/ 25.5 ± 3.8	BASDAI≥4	mNY1984	7	CNKI
Zhao and Li (106)	2013	China	21/20	Th17; Treg; Th17/Treg ratio	nr/26 ± 8	BASDAI≥5	mNY1984	5	CNKI
Zhao et al. (107)	2011	China	14/18	Treg	26.4 ± 6.1/ 28.2 ± 9.4	nr	mNY1984	5	Medline; PubMed
Zhao et al. (108)	2009	China	30/30	CD4+T; CD8+T	nr	nr	mNY1984	5	CNKI
Zhong and Ma (109)	2014	China	78/30	Th1; Th2; Th17	nr	nr	mNY1984	6	CNKI
Zhu et al. (110)	2017	China	42/42	CD4+T	nr	nr	nr	3	CNKI
Zhu et al. (111)	2016	China	30/30	NK	nr	nr	mNY1984	5	CNKI
Zhu et al. (112)	2000	China	14/7	Th1; Th2; Th1/Th2 ratio	nr	nr	mNY1984	4	CNKI

AS, ankylosing spondylitis; HC, healthy control; nr, not reported; BASDAI, Bath Ankylosing Spondylitis Activity Disease Activity Index; ASDAS: Ankylosing Spondylitis Disease Activity Score; mNY1984: 1984 Modified New York AS Criteria; ASAS2009: 2009 Assessment of SpondyloArthritis international Society (ASAS) Criteria.

proportion between AS patients and healthy controls [4.43, (-2.41,11.26), p<0.01]; however, the proportion of CD4+ T cells was significantly elevated [3.32. (1.21,5.43), p<0.01]. When examining the subsets of the CD4+ T cells, we identified significant increases in the proportion of Th17 cells[1.49, (1.03,1.65), p<0.01], Tfh cells[3.85, (0.31,7.38), p<0.01] and Th1/Th2 ratio[1.02, (0.39,1.65), p<0.01], while the proportion of Tregs was significantly decreased[-0.43, (-0.71,-0.15), p<0.01]. However, sensitivity analysis indicated that the significantly lower proportions of Tfh cells could be insignificant by omitting either Long et al, or Wu et al. No significant difference was found in the level of Th1, Th2 cells.

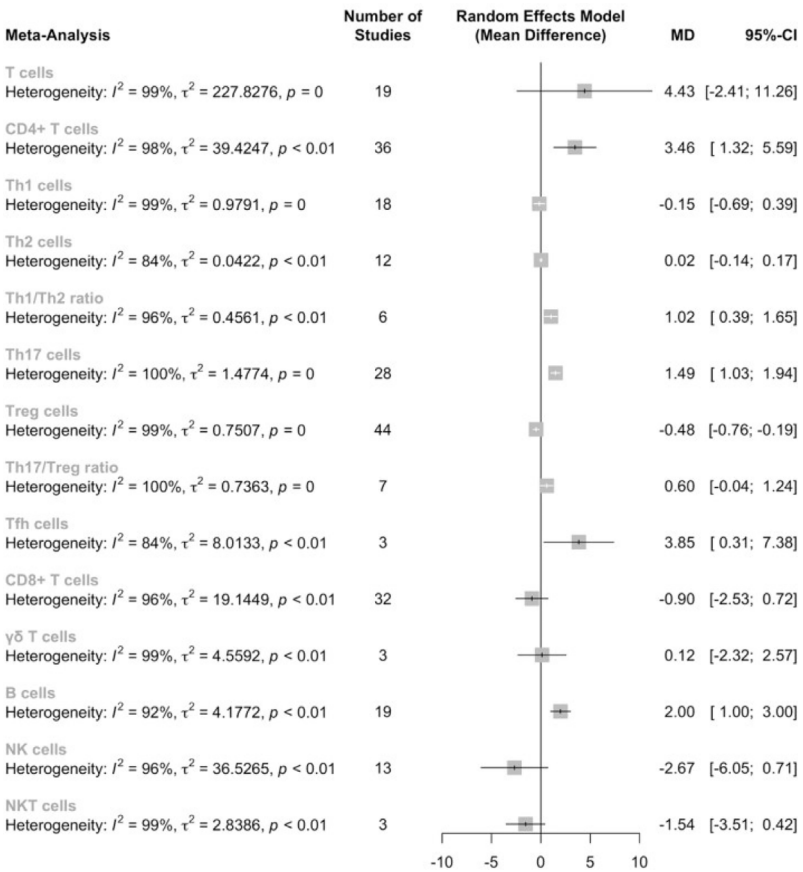

FIGURE 2 | Proportions of major lymphocyte subsets in the peripheral blood of AS patients.

Noteworthy, Th17/Treg ratio was increased but did not reach statistical significance [0.60, (-0.04,1.24), p<0.01]. According to the sensitivity analysis, if omitting Wang et al, the Th17/Treg ratio could be significantly elevated (See **Supplemental Material 2**).

Considering that the proportions of these lymphocytes were compared to PBMCs, T cells or CD4+ cells, we deemed it necessary to conduct subgroup analysis based on the level of comparisons. Subgroup analysis revealed that Th17 cells were increased on all the levels of PBMC, T cells and CD4+ cells (**Figure 3**), while Tregs were only significantly decreased on the level of PBMC (**Figure 4**). Still no significant difference was found in the proportions of Th1 and Th2 cells on all levels (**Figures 5**, **6**). On the other hand, due to the heterogeneity of the markers used to define Tregs, we also conducted a subgroup analysis of Tregs (**Table 2**). Results showed that Tregs defined by "CD4+CD25+FoxP3+", "CD4+CD25+CD127low"or "CD4+CD25+CD127-"were significantly downregulated. No significant difference was detected in the proportions of CD8+ T cells and γδ T cells.

Subgroup analysis further suggested that T cells were significantly elevated in patients with high disease activity, and that CD4+ T cells were still significantly increased in AS patients strictly defined by 1984 modified New York criteria. Furthermore, the proportion of Th17 cells remained elevated regardless of the classification criteria or disease activity, indicating the robustness of this result. Tregs were only significantly decreased in AS patients strictly defined by 1984 modified New York criteria. Intriguingly, though previous analysis failed to detect any alterations of Th1 proportions, subgroup analysis revealed that the Th1 lineage was elevated in AS patients with high disease activity, Still no alterations were observed in the proportions of Th2 cells and CD8+ T cells in AS patients (See **Supplemental Material 2**).

Egger's tests showed that there was no obvious publication bias in all the subgroups of lymphocytes (**Table 3**).

Proportion of B cells

According to the results of the meta-analysis, the proportion of B cells was significantly increased [2.00, (1.00,3.00), p<0.01]. Egger's test found no publication bias in this result (**Table 3**). Sensitivity analysis indicated that this result was robust.

Proportions of NK Cells and NKT Cells

No significant difference was found in the proportions of NK cells [-2.67, (-6.05,0.71)] and NKT cells[-1.54, (-3.51,0.42)].

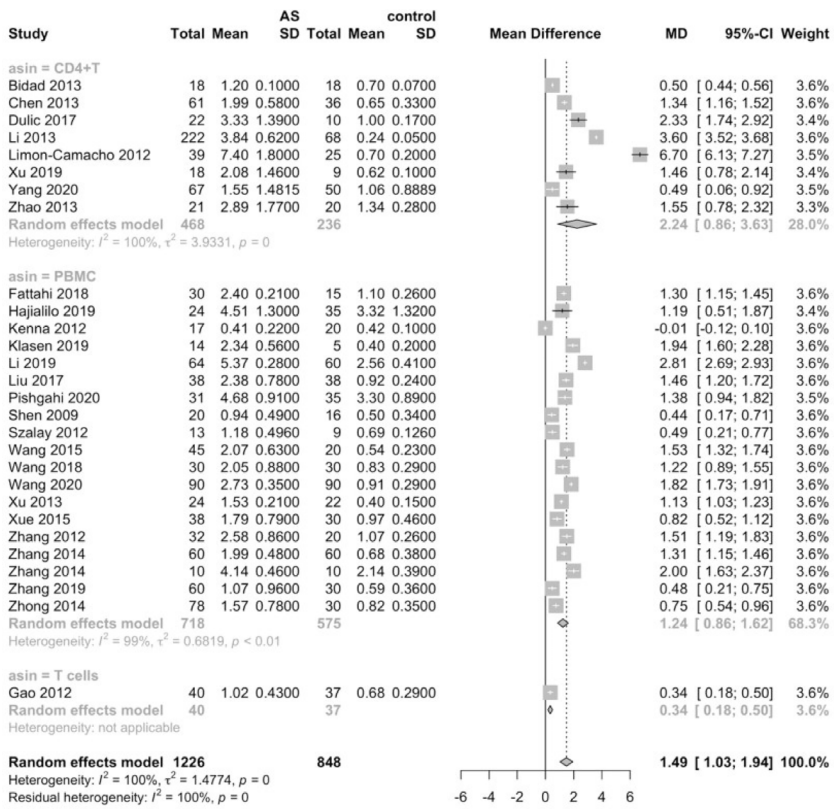

FIGURE 3 | Proportions of Th17 cells among PBMCs, T cells and CD4+ cells.

Subgroup analysis based on classification criteria and disease activity still failed to detect any differences in the proportions of NK cells between AS patients and healthy controls (See **Supplemental Material 2**). Egger's test found no publication bias in this result (**Table 3**).

Results of the sensitivity analyses can be found in the **Supplemental Material 2**.

DISCUSSION

This is the first meta-analysis to systemically examine the skewing of functional subgroups of lymphocytes, encompassing the major lymphocyte subsets, namely T cells, B cells, NK cells and NKT cells. Previous meta-analyses done by Li et al. and Lai et al. focused on regulatory T cells, arriving at the conclusion that the proportions of Tregs are significantly lower in both PBMCs and CD4+ T cells in patients with AS, though the markers used to define Tregs may have an impact on the proportions of Tregs (113, 114). In line with the results of previous studies, our study further confirmed that the levels of Tregs significantly decreased in patients with AS in PBMCs.

Tregs have been recognized as the essential subgroup of lymphocytes in charge of maintaining immune homeostasis and preventing autoimmunity. Immunosuppressive cytokines such as TGF-β and IL-10 secreted by Tregs may function as a negative regulator of immune responses and down-regulate excessive inflammatory status (115). For example, IL-10 secreted by Tregs may act directly on the IL-10 receptor on Th17 cells, thereby inhibiting the expansion of the inflammatory Th17 cells, or suppress the antigen-presenting cells and eventually suppress the responses of effector T cells (116). It has been reported that in patients with active AS, Tregs in peripheral blood fail to utilize IL-2 and cannot suppress naïve T cell proliferation (117). Moreover, application of TNF-α inhibitors can restore the proportion of Tregs, and the increase in Tregs is positively correlated with the decrease in CRP levels (94). Of note, different markers have been employed to identify the subgroup of Tregs, which may exert an influence on the proportions of Tregs measured in different studies, sometimes yielding contradictory results. Initially, Tregs is defined as CD4+CD25+, yet it was disputed since CD25 may also be expressed on cells without regulatory functions (118). Afterwards, the intracellular transcription factor (FOXP3) was proved to be exclusively expressed in Tregs and indispensable in the development of Tregs (119). The most common marker used to identify Tregs currently is CD4+CD25highCD127low or CD4+CD25highCD127-, of which CD127 is considered to be down-regulated on Tregs (113, 120). In our meta-analysis, we discovered that merely CD4+CD25+ did not produce significant outcomes regarding the proportions of Tregs, while Tregs defined by CD4+CD25+FoxP3+ and CD4+CD25+CD127low

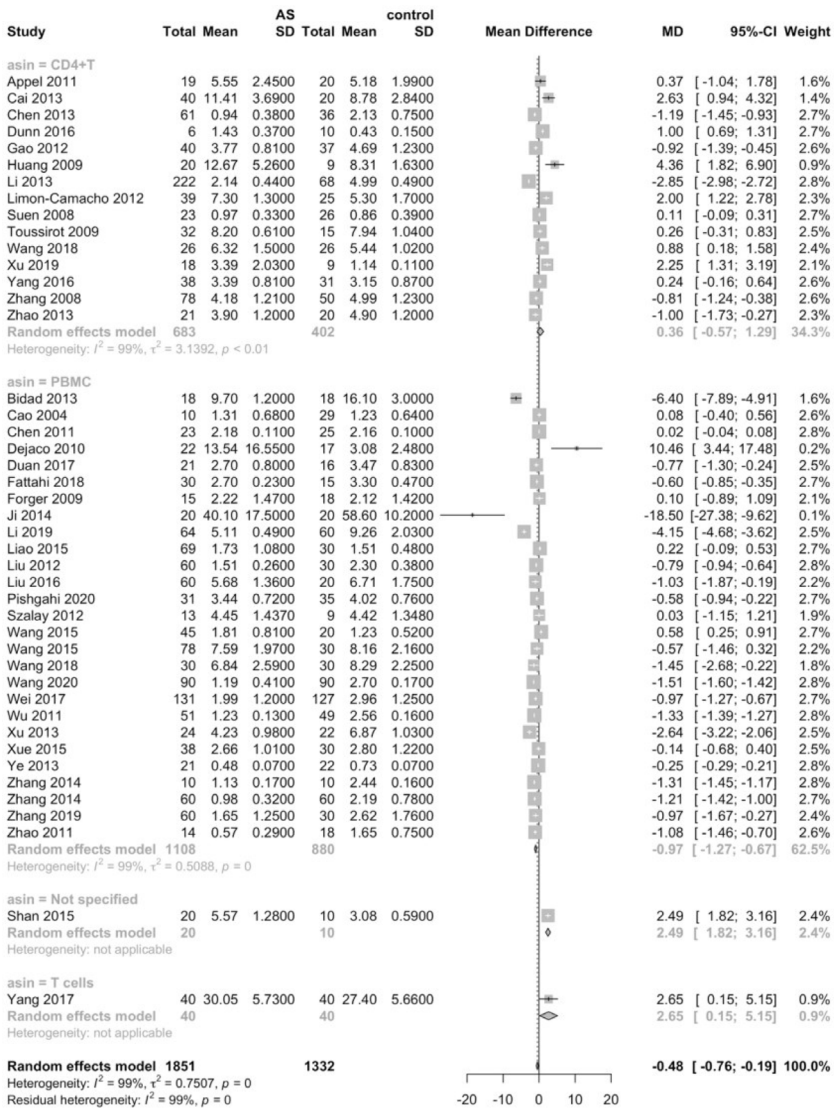

FIGURE 4 | Proportions of Tregs among PBMCs, T cells and CD4+ cells.

or CD4+CD25+CD127- were significantly lowered. This result of the subgroup analysis indicated that the CD127 could be a specific marker when trying to identify Tregs.

Upon the discovery of IL-23/IL-17 axis, the Th17 cells are moving center stage in the research of pathogenesis of spondyloarthropathies (121, 122). It has been widely acknowledged that enthesitis is the hallmark of spondyloarthropathies including AS, and recent research revealed that enthesitis is likely to be driven by the IL-23/IL-17 axis (123). IL-23, produced by myeloid cells either enthesis-resident or tissue infiltrating, may bind to the IL-23 receptors on Th17 cells as well as other lymphoid populations, and the activated Th17 cells can secrete IL-17, a powerful pro-inflammatory cytokine (123). Of the IL-17 family, IL-17A/IL-17F may act on stromal cells and other lymphocytes, which initiates the inflammatory process (17). It has also been reported that IL-17A may mediate bone damage by inducing the expression of RANK on the cell surface of osteoclasts, while also increasing the production of RANKL from mesenchymal stem cells (124). Apart from IL-17, Th17 lymphocytes are known to produce other pro-inflammatory mediators, such as IL-22, GM-CSF and TNF (125). All these studies further cemented the significance of Th17 cells in the pathogenesis of enthesitis, and, in a bigger picture, spondyloarthropathies. Our study substantiated that the levels of Th17 cells were significantly elevated, adding more concrete evidence to the critical role Th17 lineage plays in the pathogenesis in AS. Subgroup analysis further verified the robustness of this result, since Th17 cells were elevated on all levels of comparison, regardless of the classification criteria applied or disease activity of the patients.

In addition to Th17 cells, γδ T cells may also participate in the IL-23/IL-17 axis (123). γδ T cells are a specific population of T

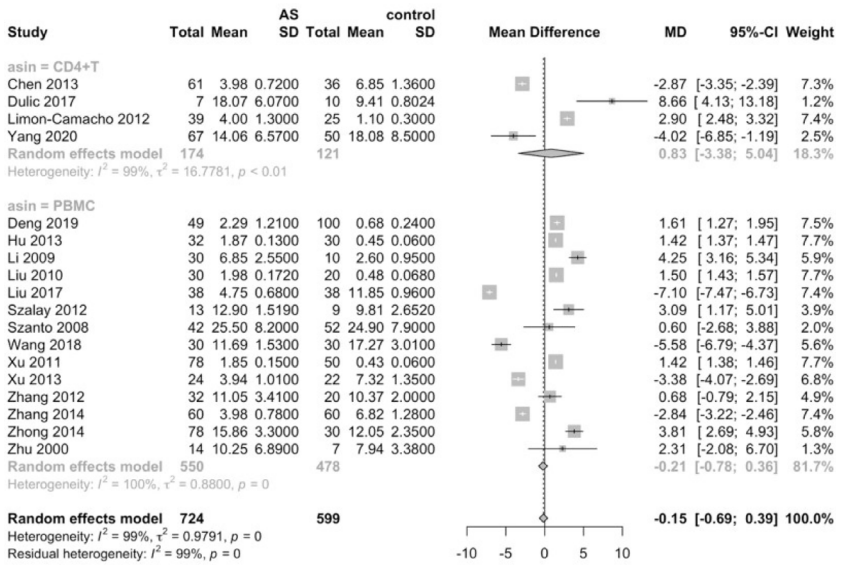

FIGURE 5 | Proportions of Th1 cells among PBMCs and CD4+ cells.

lymphocytes characterized by the highly diverse TCR on the cell surface, formulating TCR repertoire (126). Studies show that there is a 3-fold increase in the proportions of IL-23R-positive γδ T cells in AS patients, and such γδ T cells are also heavily skewed towards IL-17 production (48). Another study shows that IL-23R+ γδ T cells are the main producers of IL-17 in a mice model (127). More recent studies have revealed that IL-17 may also be produced in an IL-23-independent fashion (128). Therapies targeting IL-23 have failed in patients with SpA, while the downstream inhibition of IL-17 by IL-17A inhibitor Secukinumab and IL-17A/IL-17F inhibitor bimekizumab has yielded promising results in patients with SpA (129–131). Such phenomenon pointed to a possible pathway that IL-17 may be secreted without the stimulus of IL-23. It has been proved that γ/δ T cells may still secrete IL-17 despite the homozygous deletion of IL-23R (128). However, our study failed to recognize any alteration in the levels of γ/δ T cells. It could be attributed to the limited number of studies included, or that it was not the elevated number but the hyperactivity that was to blame for the IL-23 independent IL-17 secretion. Furthermore, RORγt+ iNKT cells were also reported to be able to secrete IL-17 with and without the effect of IL-23 (132).

In the meanwhile, the Th1/Th2 polarization of T helper cells is also a widely researched area in the immunity of AS (9). Th1 cells are known to mount immune responses against intracellular pathogens *via* secretion of IFNγ, which acts as a macrophage-activating factor (133). In addition to IFNγ, Th1 cells are also capable of producing IL-2, IL-10 and TNF-α, many of which

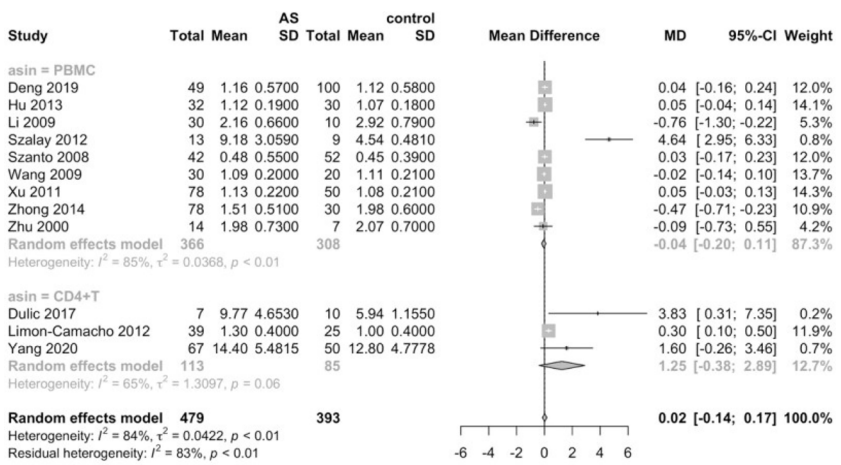

FIGURE 6 | Proportions of Th2 cells among PBMCs and CD4+ cells.

TABLE 2 | Egger's tests by different lymphocyte subsets.

Lymphocyte subset	t	df	p-value
T cells	0.49132	17	0.6295
CD4+ T cells	-1.9812	34	0.0557
Th1 cells	-1.6584	16	0.1167
Th2 cells	0.82077	10	0.4309
Th1/Th2 ratio	-0.55844	4	0.6063
Th17	0.23055	26	0.8195
Tregs	-0.086137	42	0.9318
Th17/Tregs	0.71323	5	0.5076
Tfh	0.16592	1	0.8953
CD8+ T cells	1.1247	30	0.2696
γδ T cells	-0.55802	1	0.676
B cells	1.7184	17	0.1039
NK cells	1.812	11	0.09735
NKT cells	-1.51	1	0.3724

TABLE 3 | Subgroup analysis of Tregs proportions based on markers.

Treg definition	Number of studies (n)	SMD	95%CI	(%)	P
CD4+CD25+	3	1.11	(-1.77,3.98)	97	<0.01
CD4+CD25HI	6	0.23	(-0.26,0.71)	72	<0.01
CD4+FoxP3+	3	-1.32	(-6.12,3.49)	98	<0.01
CD4+CD25+FoxP3+	12	-0.75	(-1.28,-0.22)	99	<0.01
CD4+CD25+CD127LO	6	-2.18	(-3.55,-0.81)	97	<0.01
CD4+CD25+CD127-	4	-0.74	(-0.91,-0.57)	8	0.36
CD4+CD25HIFoxP3+	2	0.55	(-.33,1.42)	95	<0.01
CD4+CD25 +CD127LO/-	4	-0.57	(-1.46,0.32)	84	<0.01
CD4+CD25+FoxP3 +CD127-	1	0.88	(0.18,1.58)	/	/
Not specified	1	-1.51	(-1.6,-1.42)	/	/

participate in the inflammatory process (134, 135). Th2 cells, on the other hand, mainly assist in the humoral immune response (136). Cytokines secreted by Th2 cells include IL-4, IL-5 and IL-13, which facilitate the isotope switching of antibodies, mucus secretion and eosinophilia (137). Data concerning the Th1/Th2 skewing in the peripheral blood of AS patients has been highly inconsistent. Some studies reported that T helper cells in AS were skewed towards Th1 lineage suggesting that Th1 cells contributed to the excessive inflammation (56), while others failed to observe such elevations in proportions of Th1 cells (73). Our meta-analysis concluded that there was no significant alteration in the proportions of Th1 and Th2 cells overall, yet subgroup analysis revealed a significant increase in the percentages in AS patients with high disease activity, indicating that the Th1 lineage might be relevant in the acute phase. Meanwhile, the Th1/Th2 ratio was also significantly elevated. More recent research provides evidence that the plasticity of Th17 cells allows this subset of CD4+ T cells to partly assume phenotype of Th1 lineage or Th2 lineage, blurring the boundaries between Th1, Th2 and Th17 cells. It has been argued that the categorical dichotomy of Th1/Th2 should be rendered obsolete.

Another intriguing finding of our study is that the proportions of Tfh cells and B cells are significantly elevated in the peripheral blood of AS patients. Both Tfh cells and B cells participate in humoral immunity (136). After migrating to the B cell follicles, CD40L expressed on the cell surface of Tfh cells may interact with the CD40 on B cells serving as a stimulus signal, thereby facilitating the formation of germinal center, differentiation of B cells and ultimately the production of antibodies (63, 138). The relevance of humoral immunity in the pathogenesis of ankylosing spondylitis has long been underestimated, since no auto-antibody is universally acknowledged as the specific marker of AS (11). Although several studies have put forward that anti-CD74 antibody may serve as a potential biomarker for AS, its diagnostic utility awaits further confirmation (139). Another possible mechanism of the B lymphocyte involvement in the pathogenesis of AS is that B lymphocytes might mediate bone destruction through production of RANKL, as was previously reported in rheumatoid arthritis (140), How Tfh cells and B cells are involved in the pathogenesis of AS still requires more research.

As a pivotal component in the innate immunity, NK cells possess cytotoxic activity and the ability of producing pro-inflammatory cytokines, such as IFNγ (141). There is mounting evidence that NK cells, with its expression of KIR superfamily on the cell surface, may contribute to the pathogenesis of ankylosing spondylitis. Different KIRs may interact with HLA alleles in various forms, creating sophisticated genotypes of NK cells. It is hypothesized that the HLA-Bw4 group of alleles, notably HLA-B27, may bind to the KIR antigens with varying affinities, displaying inhibitory or stimulatory activities through downstream signal pathways (141–143). In particular, being an inhibitory receptor, KIR3DL2 ligation with HLA-B27 may inhibit apoptosis of NK cells and protect them from activation-induced cell death (142). However, studies regarding the frequency of NK cells in the peripheral blood of AS patients have been highly inconsistent. Azuz-Lieberman et al. found that AS patients have significantly higher percentages of NK cells in PB, while the inhibitory receptor CEACAM1 is highly expressed on the surface indicating suppressed function of NK cells (144). Another study also confirmed a higher frequency of NK cells expressing KIR3DL1 in SpA patients, with an impaired IFN-γ intracellular production in stimulated NK cells (145). However, such

alteration of NK cell proportions was not observed in another study by Park, et al. (146) A more recent study found that NK cells in the peripheral blood was significantly reduced (84). Due to the inconsistencies of the data, our study failed to recognize a shift in the proportions of NK cells in the peripheral blood of AS patients.

There is no denying that there were some limitations to this study. Though being an all-encompassing meta-analysis attempting to include all the major subsets of lymphocytes, this study failed to conduct a more in-depth look into the more subtle minor subsets of lymphocytes, such as Th22 in CD4+ T cells, Tc1 and Tc2 in CD8+ T cells, Bregs, naïve B cells and memory B cells in the B cell lineage. This study originally intended to include Th22 subset in the meta-analysis, considering recent discovery that Th22 cells might have the capacity to promote osteoclast differentiation though production o IL-22 (147). To our chagrin, there were not enough studies to conduct an appropriate analysis. Second, lymphocytes may assume complicated phenotypes by expressing various antigens on the cell surface. Therefore, this crude classification of lymphocytes may not be adequate to explain the exact shifting of immune system in AS patients. However, further investigation was impeded by the insufficiency of the data. Third, there was notable heterogeneity in the studies considering the selected patients might have undergone different treatments and might be in different phases, it might be more appropriate to look into the effects of different treatments on the lymphocyte subsets. Moreover, this study only targeted lymphocytes in the peripheral blood, which could not adequately reflect the inflammatory status of the tissue.

In conclusion, our meta-analysis concluded that CD4+ T, Th17, Tfh and B cells were significantly elevated in the peripheral blood of AS patients, while Tregs were significantly reduced. Our study further cemented the understanding that the nature of ankylosing spondylitis is a hybrid of innate immunity and acquired immunity dysfunction.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

JG conceived of the presented idea. DL and BL conducted the literature search, while DL performed the analytic calculation and drafted the final version of this manuscript. CL settled any disagreements concerning the inclusion of literature. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.696973/full#supplementary-material

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Active Inflammatory and Chronic Structural Damages of Sacroiliac Joint in Patients With Radiographic Axial Spondyloarthritis and Non-Radiographic Axial Spondyloarthritis

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Objective: Evaluate the MRI evidence of active inflammatory and chronic structural damages in radiographic axial spondyloarthritis (r-axSpA) and non-radiographic axial spondyloarthritis (nr-axSpA).

Methods: A retrospective review of 253 patients who underwent sacroiliac joint (SIJ) MRI between June 2014 and December 2019 was performed. MRI images including short tau inversion recovery scan and T1-weighted spin echo scans were assessed using the Spondyloarthritis Research Consortium of Canada (SPARCC) score and SPARCC MRI SIJ structural score by two independent readers.

Results: Higher mean score of inflammatory (SPARCC) was seen in r-axSpA patients when compared with nr-axSpA patients (8.08 *vs* 4.37, $P<0.05$). Frequencies of MRI structural lesions in r-axSpA patients and nr-axSpA patients were as follows: erosion (65.84 *vs* 88.23%, $P=0.002$), backfill (33.17 *vs* 13.73%, $P<0.001$), fat metaplasia (79.21 *vs* 60.78%, $P=0.01$), and ankylosis (37.13 *vs* 1.96%, $P<0.001$). Patients with r-axSpA had a higher mean score for fat metaplasia (8.93 *vs* 4.06, $P=0.0003$) and ankylosis (4.49 *vs* 0.04, $P<0.001$).

Conclusion: More active inflammatory and chronic structural damages except for erosion were seen in r-axSpA patients than nr-axSpA patients, while higher percentage of nr-axSpA patients presented with erosion in MRI.

Keywords: ankylosing spondylitis, axial spondyloarthritis, inflammation, structural damage, MRI

INTRODUCTION

Spondyloarthritis (SpA) is a group of inflammatory diseases mainly affecting the sacroiliac (SI) joint and spine. Axial spondyloarthritis (axSpA) includes non-radiographic axial spondyloarthritis (nr-axSpA) and radiographic axial spondyloarthritis (r-axSpA); the latter one is also termed ankylosing spondylitis (AS). Nr-axSpA and r-axSpA share some similarities such as disease

activity (defined by Bath Ankylosing Spondylitis Disease Activity Index—BASDAI), physical function, prevalence of HLA-B27 positivity, and comorbidity burden (1–3), while there are several differences including disease course, gender predilection, and levels of inflammatory markers (4).

MRI is an objective measurement detecting acute inflammatory and chronic structural changes recommended by the Assessment of SpondyloArthritis International Society (5). Bone marrow edema (BME), capsulitis, and subligamentous enthesitis are defined as active inflammatory changes in SpA according to the update of the ASAS MRI working group (6). Chronic structural changes including subchondral sclerosis, erosion, backfill, fat metaplasia, and ankylosis are present better and earlier in CT (7) or MRI (8) than radiographs.

Inflammatory and chronic structural changes are objective signs of axSpA. Previous studies indicated that sacroiliitis on radiographs is usually bilateral and symmetric in AS (9). However, the differences of structural damages between r-axSpA and nr-axSpA patients on MRI are limited. The aim of this study was to investigate whether there are differences between Chinese r-axSpA and nr-axSpA patients in inflammatory and chronic structural lesions on MRI, and clinical factors related to MRI changes.

METHODS

Patients

A retrospective study was performed, and patients with MRI and radiographic imaging of the sacroiliac joint and diagnosed with axSpA between June 2014 and December 2019 were included. The local ethics committee waived approval because of the retrospective nature of this study. The patients provided their written informed consent to anonymize the use of relative data in further study according to daily clinical practices in this department. Demographic characteristics and inflammatory indicators such as C-reactive protein (CRP) and Erythrocyte Sedimentation Rate (ESR) are collected.

MRI Protocol and Assessment

All MR images were acquired on a 1.5 Tesla or 3 Tesla MR scanner in a coronal plane titled parallel to the long axis of the SI joint with 3–4 mm slice thickness and 12–15 slices acquired. Short-tau inversion recovery (STIR) sequences were used for the assessment of inflammatory lesion. The T1-weighted MRI sequences were used for the assessment of chronic/structural changes. The Spondyloarthritis Research Consortium of Canada SPARCC MRI scoring system was used to assess inflammation and structural damages in SI joint. SPARCC SIJ scores were based on the measurement of six consecutive slices, with the largest score of 12 for edema, intensity, and depth per slice, and a total score of 0–72 was calculated. SPARCC SI structural lesion score (SSS) (10) was used to assess the structural lesions, and four kinds of lesions were assessed based on five consecutive slices through the SIJ. Fat metaplasia is defined as an increased signal in bone marrow on T1WSE, and the lesion has to demonstrate homogeneous signal with more than 1 cm in depth from the joint surface. Erosion is defined as the full-thickness loss of the dark appearance of either iliac or sacral cortical bone at its anticipated location and loss of the normal bright appearance of adjacent bone marrow. Backfill is defined as complete loss of iliac or sacral cortical bone at its anticipated location and increased signal that is clearly demarcated from adjacent normal marrow by irregular dark signal reflecting sclerosis at the border of the eroded bone. Ankylosis is defined as bone marrow signal on T1WSE sequences extending between the sacral and iliac bone marrow with a full-thickness loss of the dark appearance of the iliac and sacral cortical bone. The presence/absence of lesions is scored using an online data entry system in SIJ quadrants (fat, erosion) or halves (backfill, ankylosis) with a scoring range of 0–40 for quadrants lesions and 0–20 for halves lesions. Examples of these inflammatory and structural damages are shown in **Figure 1**. Images were assessed by two radiologists independently, who were blinded to all patient data. The mean score of two radiologists was used for analysis. For SPARCC SIJ score, two readers had to discuss and reach consensus if a difference of >3 points existed (11). A SPARCC SSS >0 was required from both readers for identification of a structural lesion of erosion, backfill, fat metaplasia, or ankylosis (12). The before image evaluation, a training session including 20 test images, was carried out by an expert rheumatologist. A score ≥2 for SI joint bone marrow edema (BME) was considered positive MRI evidence of inflammation, and this cutoff has been validated in the literature (3, 13).

Statistical Analysis

Statistical analyses were performed using Stata Version 15.0. Summary statistics such as frequency, percentage, mean, and standard deviation were used to analyze demographic characteristics and inflammatory and structural changes of axSpA patients. T-test was used to evaluate the difference of demographic characteristics and MRI scores between AS and nr-axSpA patients. Spearman correlations were used to determine the correlation between SPARCC scores and clinical factors. Then we conducted multivariate stepwise regression analyses to determine the best significant predictors for inflammatory and structural lesions. All tests were two-sided, and a significance level of $P < 0.05$ was assumed. Interobserver reliability for imaging scores was assessed using the intraclass correlation coefficient (ICC). The ICC value of <0.4 was designed as fair, ≥0.4 but <0.6 as moderate, ≥0.6 but <0.8 as good, ≥0.8 but <0.9 as very good, and ≥0.9 as excellent.

RESULTS

Demographic

A total of 253 patients with a clinical diagnosis of axSpA were included for analysis during June 2014 and December 2019 in the third affiliated hospital of SUN YAT-SEN University. Fifty-one patients were classified as nr-axSpA, while others fulfilled the diagnosis of r-axSpA. The average age of patients was 31.74 years with a mean disease duration of 7.91 years.

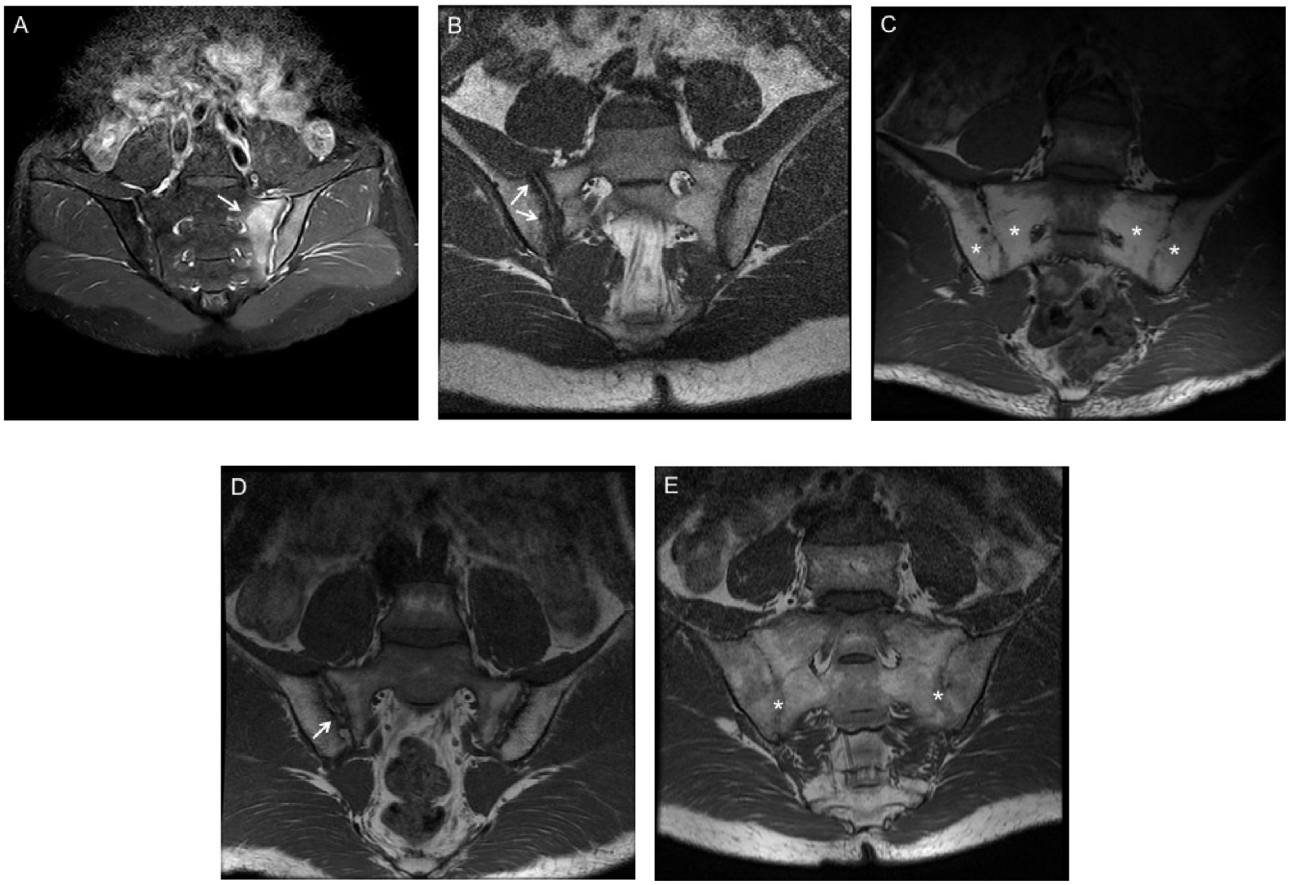

FIGURE 1 | MRI findings of inflammatory and structural damage of SpA. **(A)** Bone marrow edema (BME) at the left side of sacroiliac joints (white arrows) in Coronal oblique STIR sequence. **(B)** Erosion of the right iliac bone on T1 weighted spin echo MRI (white arrows). **(C)** Fat metaplasia at the bilateral sacroiliac joints on T1 weighted spin echo MRI (white stars). **(D)** Backfill of the right iliac bone on T1 weighted spin echo MRI (white arrows). **(E)** Ankylosis of the bilateral sacroiliac joints on T1 weighted spin echo MRI (white stars).

A total of 87.35% (n=221) of patients were HLA-B27 positive, and 79.45% of them were male. The mean BASDAI and BASFI scores were 4.31 ± 2.25 and 2.19 ± 2.27 respectively, and 33.2% (n=84) of patients had used tumor necrosis factor inhibitor (TNFi) in the recent 3 months. Longer disease duration, higher positive rate of HLA-B27, and worse function, as measured by BASFI, were seen in r-axSpA group. The disease activity markers of BASDAI, ASDAS, and SPARCC MRI SIJ inflammation were significantly higher in r-axSpA group. No significant differences in other demographic features were noted between the groups (**Table 1**).

Inflammatory and Structural Changes on MRI

Higher SPARCC score was seen in r-axSpA patients than in nr-axSpA patients. Of the 253 patients evaluated, 117 (46.25%) had no BME seen on MRI. There was no significant difference in percentage of patients with BME between r-axSpA and nr-axSpA groups. Clinical characteristics such as age, sex, BASDAI, and CRP did not differ between patients with and without BME. There was significant difference in the mean score of erosion, backfill, and ankylosis between patients with and without SIJ BME (**Table 2**). Relative frequencies of MRI structural lesions in patients with *versus* without SIJ BME were as follows: erosion (78/136, 57.35% *vs* 100/117, 85.47%; $P<0.001$), backfill (23/136, 16.91% *vs* 51/117, 43.59%; $P<0.001$), fat metaplasia (93/136, 68.38% *vs* 98/117, 83.76%; $P=0.005$), and ankylosis (50/136, 36.76% *vs* 26/117, 22.22%; $P=0.01$).

The majority of patients had a score of >0 for structural damages for at least one of fat metaplasia, erosion, backfill, and ankylosis in the SI joint. A total of 242 (95.65%) patients had ≥1 structural lesion on MRI comprising erosion 71.54%, backfill 29.24%, fat metaplasia 75.49%, and ankylosis 30.04%. In SSS structural scores, significant difference was found between r-axSpA and nr-axSpA groups in fat metaplasia, backfill, and ankylosis (**Table 2**). Frequencies of MRI structural lesions in r-axSpA patients and nr-axSpA patients were as follows: erosion (65.84 *vs* 88.23%, $P=0.002$), backfill (33.17 *vs* 13.73%, $P<0.001$), fat metaplasia (79.21 *vs* 60.78%, $P=0.01$), and ankylosis (37.13 *vs* 1.96%, $P<0.001$).

TABLE 1 | Demographic features of included patients.

Characteristics	All patients (n=254)	AS (n=202)	nr-axSpA (n=51)	*P*-value
Age (years)	31.74±9.14	31.7±9.25	31.88±8.77	0.9
Male patients, n (%)	201 (79.45)	164 (81.19)	37 (72.55)	0.18
Disease duration (years)	7.91±6.56	8.68±6.50	4.94±5.95	0.0002
HLA-B27, n (%)	221 (87.35%)	182 (90.1)	39 (76.47)	0.02
Family history, n (%)	60 (23.72)	51 (25.25)	9 (17.65)	0.35
CRP (mg/dl)	18.66±23.99	20.05±25.18	13.19±17.75	0.06
ESR (mm/H)	26.07±24.13	26.54±23.22	24.18±27.58	0.53
BASDAI (0-10)	4.31±2.25	4.53±2.26	3.43±2.02	0.002
BASFI (0-10)	2.19±2.27	2.38±2.29	1.44±0.05	0.009
ASDAS-CRP	2.98±1.17	3.14±1.13	2.37±1.13	<0.000
Use of TNFi, n (%)	84 (33.2%)	63 (31.2%)	21 (41.2%)	0.19

Data were presented as mean±SD unless specifically indicated. HLA-B27, Human leukocyte antigen-B27; CRP, C-Reactive Protein; ESR, Erythrocyte Sedimentation Rate; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; BASFI, Bath Ankylosing Spondylitis Functional Index; BASMI, Bath Ankylosing Spondylitis Metrology Index; ASDAS, Ankylosing Spondylitis Disease Activity Score; TNFi, Tumor necrosis factor inhibitor.

TABLE 2 | MRI inflammatory and structural damages.

Characteristics	All patients (n=253)	AS (n=202)	nr-axSpA (n=51)	BME≥2 (N=136)	BME<2 (N=117)
SPARCC (0-72)	7.33 ± 12.02	8.08 ± 12.27*	4.37 ± 10.55	–	–
Fat metaplasia (0-40)	7.95 ± 8.74	8.93 ± 9.15*	4.06 ± 5.33	7.56 ± 9.34	8.41 ± 7.98
Erosion (0-40)	5.01 ± 6.41	5.13 ± 6.79	4.51 ± 4.60	3.36 ± 5.39$^{\eta}$	6.93 ± 6.97
Backfill (0-20)	1.51 ± 3.33	1.71 ± 3.35*	0.75 ± 3.19	0.68 ± 2.19$^{\eta}$	2.48 ± 4.11
Ankylosis (0-20)	3.59 ± 6.72	4.49 ± 7.25*	0.04 ± 0.28	5.37 ± 7.98$^{\eta}$	1.65 ± 4.10

*SPARCC, the Spondyloarthritis Research Consortium of Canada; AS, ankylosing spondylitis; nr-axSpA, non-radiographic axial spondyloarthritis; BME, bone marrow edema. *Significant difference was found between AS and nr-axSpA patients.*$^{\eta}$*Significant difference was found between patients with and without BME.*

Correlations Between MRI Inflammatory and Structural Lesions

Significant correlation was found between SPARCC score and age (−0.21, P <0.01), sex (−0.15, P =0.02). In multivariable stepwise regression analysis, age (−0.28, P =0.001) and HLA-B27 (4.53, P =0.046) were found significantly associated with SPARCC score.

Table 3 shows factors associated with SSS scores. Fat metaplasia was associated with age, sex, and BASDAI, and only BASDAI (β: −0.56, P =0.02) remained significant in multivariable analysis. For erosion, significant association was found with BASDAI (β: −0.88, P <0.01) and SPARCC (β: 0.18, P <0.01) in multivariable analysis. Backfill was associated with SPARCC (β: 0.05, P <0.01) in multivariable analysis. For ankylosis, age, sex, HLA-B27, disease duration, CRP, and SPARCC were found significantly associated.

TABLE 3 | Factors associated with structural damages on MRI.

	Fat metaplasia		Erosion		Backfill		Ankylosis	
	Univariable Spearmen's rho, P value	Multivariable β (95% CI)	Univariable Spearmen's rho, P value	Multivariable β (95% CI)	Univariable Spearmen's rho, P value	Multivariable β (95% CI)	Univariable Spearmen's rho, P value	Multivariable β (95% CI)
Age	**0.12, P=0.046**	NA	-0.08, NS	NA	-0.07, NS	NA	-0.02, NS	**-0.17 (-0.27, -0.07)**
Sex	-0.02, NS	NA	0.10, NS	NA	-0.007, NS	NA	**-0.15, P=0.02**	**-2.14 (-3.83, -0.44)**
HLA-B27	0.11, NS	3.09 (-0.13, 6.30)	-0.02, NS	NA	0.09, NS	NA	**0.15, P=0.02**	**2.50 (0.16, 4.83)**
Disease duration	**0.14, P=0.02**	NA	**-0.12, P=0.48**	NA	-0.02, NS	NA	**0.26, P<0.001**	**0.27 (0.13, 0.41)**
BASDAI	**-0.16, P<0.01**	**-0.88 (-1.21, -0.54)**	**-0.39, P<0.01**	**-0.88 (-1.21, -0.54)**	-0.10, NS	NA	0.12, NS	NA
CRP	0.04, NS	NA	**-0.13, P=0.04**	NA	-0.02, NS	NA	**0.28, P<0.001**	**0.05 (0.02, 0.08)**
ESR	-0.05, NS	NA	-0.06, NS	NA	0.006, NS	NA	0.12, NS	NA
ASDAS	-0.07, NS	NA	**-0.31, P<0.01**	NA	-0.07, NS	NA	**0.22, P<0.001**	NA
SPARCC	0.09, NS	NA	**0.36, P<0.01**	**0.18 (0.12, 0.24)**	**0.31, P<0.001**	**0.05 (0.01, 0.09)**	**-0.22, P<0.01**	**-0.17 (-0.23, -0.10)**

HLA-B27, Human leukocyte antigen-B27; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; BASFI, Bath Ankylosing Spondylitis Functional Index; CRP, C-Reactive Protein; ESR, Erythrocyte Sedimentation Rate; ASDAS, Ankylosing Spondylitis Disease Activity Score; SPARCC, the Spondyloarthritis Research Consortium of Canada; NS, not significant; NA, not available.

Bold values means significant difference.

Interobserver Reliability

ICC was computed to test interobserver reliability. Excellent correlation was achieved for rating of SPARCC score and ankylosis (ICC scores of 0.98 [95% CI 0.93–0.99] and 0.95 [95% CI 0.91–0.98], respectively). Good correlation was achieved for identification of erosion, fat metaplasia, and backfill with ICC scores of 0.85 (95% CI 0.71–0.93) for fat metaplasia, 0.87 (95% CI 0.75–0.94) for erosion, and 0.74 (95% CI 0.52–0.87) for backfill.

DISCUSSION

The results of this study demonstrate that structural lesions on MRI may occur in early stage of SpA (nr-axSpA) and in the absence of SIJ BME on MRI. R-axSpA patients have more SIJ inflammation on MRI, and fat metaplasia is seen more in r-axSpA than nr-axSpA patients. Our data also support that structural damages in SIJ are associated with active inflammation.

Conventional radiograph is still preferred for the analysis of SIJ structural damage and for the diagnosis of axSpA. It has been verified that T1-weighted MRI sequences were superior to conventional radiographs in the detection of erosion of SIJ in axSpA patients (14). Besides, additional lesions such as fat metaplasia, backfill, and ankylosis can be observed clearly on MRI. The distribution pattern of inflammation and structural lesions may differ between AS and nr-axSpA patients; unilateral fat metaplasia and erosion were more seen in nr-axSpA patients (15) with a small simple size. This study compared the inflammation and structural lesions in r-axSpA and nr-axSpA patients using SPARCC scoring system and analyzed factor related to MRI changes.

Fat metaplasia, one of the chronic lesions and usually observed in the bone marrow of SI joint and spine, is assumed to be an intermediate stage between active inflammation and formation of new bone (16). Prospective studies indicated that fat metaplasia and backfill are associated with the development of new ankylosis (8, 14, 16). However, the proportion of fat marrow increases over time, and a previous study reported that a very high prevalence of fat metaplasia (50.6% in the age groups less than 45years, 94.4% in patients ≥75 years) was found in the SIJ of asymptomatic patients, while erosions were extremely uncommon (17). Fat metaplasia with certain features such as a distinct border, proximity to subchondral bone, and homogeneity of T1-weighted signal, combined with other abnormalities (bone marrow edema and erosion), is assumed to be highly specific for SpA but does no help for diagnosis (18).

The relationship between inflammation, fat metaplasia, and ankylosis has been studied before. It is reported that baseline SPARCC SIJ inflammation and SIJ backfill scores independently predicted progression in SPARCC SIJ ankylosis (19). TNFi can retard not only inflammation but also structural progression rate on MRI in both short-term (11, 20) and long-term follow-up (19). It is reported that etanercept has more effect than usual care on SIJ erosion development; attaining sustained ASDAS inactive disease is relevant to the amelioration of erosion (21). Reduction of inflammation, erosion, and increase of fat metaplasia were seen in SpA patients treated with adalimumab compared with placebo group (20). Other clinical factors such as disease activity, demographics, and HLA-27 were not associated with development of MRI structural features according to previous study (22).

There are several limitations of this study. First is the retrospective nature, which is subject to inherent observation bias. Second is the interpretation of the MRI T1 sequence. The structural damages such as erosion and backfill are heterogeneous lesions, which are difficult to discern and need caution when interpreting the results. Besides, these damages could be presented in other diseases such as scoliosis and degenerative disease. Further validation of structural damages using both computed tomography and MRI is needed for better understanding.

CONCLUSIONS

This study indicated that higher inflammatory and structural damages except for erosion were seen in r-axSpA patients when compared with nr-axSpA patients. In patients without MRI SIJ BME, structural lesions are also present. Prospective study is necessary to understand the relationship between structural damages and treatments.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by third affiliated hospital of SUN YAT-SEN University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JG had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: JG and LT. Acquisition, analysis, or interpretation of data: all authors. Drafting the manuscript: LT and JG. Critical revision of the manuscript for important intellectual content: all authors. All authors contributed to the article and approved the submitted version.

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Adalimumab Therapy Restores the Gut Microbiota in Patients With Ankylosing Spondylitis

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Growing evidence suggests that the gut microbiota is involved in the initiation and progression of ankylosing spondylitis (AS). In this study, we aimed to explore the gut microbiome alterations during adalimumab therapy and verify microbiome biomarkers predicting treatment response. By evaluating the gut microbial features of 30 AS patients before and after adalimumab therapy for 6 months and 24 healthy controls, we confirmed that the microbiome was restored remarkably after 6 months of adalimumab therapy in AS patients. We then compared the baseline gut microbiome of 22 adalimumab responders with 8 non-responders, a higher abundance of *Comamonas* was revealed in the latter, although no statistical difference was found after adjusting for the false discovery rate. These results suggested that adalimumab therapy restored the gut microbiome in AS patients and indicated the utility of gut microbiome to be potential biomarkers for therapeutic evaluation. These findings provided an insight into the development of predictive tools and the establishment of precise medical interventions for clinical practice.

Keywords: adalimumab, TNF, gut microbiome, ankylosing spondylitis, biomarker

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INTRODUCTION

Ankylosing spondylitis (AS) is a chronic inflammatory disorder that affects the axial skeleton, causing characteristic inflammatory back pain that can lead to structural and functional impairments and a decreased quality of life (1). Among patients with AS, 40–60% present with subclinical intestinal inflammation, and 5–10% progress to clinical inflammatory bowel disease (IBD) (2). In recent years, substantial evidence has indicated the vital role of the gut microbiota in the initiation and progression of IBD (3). Similarly, a growing number of studies revealed perturbed gut microbiota in AS or spondyloarthritis (SpA) patients (4), and also in AS animal model typically HLA-B27 transgenic rats (5). Moreover, some altered species like *Dialister*, was related to AS disease activity (6). Recently, HLA- B27- positive healthy individuals were identified with a significantly different microbiome (7), indicating that the gut microbiota may in fact be a driver of AS.

The development of tumor necrosis factor inhibitor (TNFi) and its introduction into clinics was a milestone in the treatments for autoimmune diseases, including AS, IBD, psoriasis, and rheumatoid arthritis. It is well known that TNFi improves symptoms and inflammatory cytokine levels in patients with AS. However, it is unknown whether TNFi affects the gut microbiome due to a lack of evidence. A previous study analyzed the gut microbiome of SpA patients before and after

3 months of TNFi treatment, using stool samples (8). Only a modest difference in the alpha diversity of the gut microbiome was found, while no specific bacterial taxa were observed. But this study did not include healthy controls, so it is uncertain whether TNFi treatment was responsible for restoring the gut microbiome to a healthier status. Recently, restoration of gut microbiota composition was revealed in proteoglycan-induced AS mice after TNFi treatment (9), indicating that TNFi treatment might affect the gut microbiota.

As humanized monoclonal antibody targeting TNF, adalimumab has been successfully used to manage inflammation in AS patients in clinic, especially in those with extra-articular symptoms such as IBD and uveitis (10). It was also reported to improve symptoms and restore gut microbiota in patients with IBD (11). In addition, baseline features of the gut microbiome were valuable in predicting the treatment response to TNFi in patients with IBD (12). However, the association between the gut microbiome alteration and adalimumab treatment in AS patients is unknown.

In this study, we recruited AS patients prescribed adalimumab and healthy controls to evaluate the effect of adalimumab treatment on the gut microbiome. Further, we explored whether the gut microbiome can be used to predict the response to adalimumab treatment in AS patients.

MATERIALS AND METHODS

Populations and Sample Size

We conducted a prospective observational study between January 2017 and July 2018. Patients with AS who were initiated adalimumab therapy were consecutively recruited at the outpatient clinic of Rheumatology at the Third Affiliated Hospital of Sun Yat-sen University. The inclusion criteria were as follows: (1) age of 18 years or older, (2) fulfillment of the 1984 modified New York Criteria for AS (13), (3) inadequate improvement despite taking at least two non-steroidal anti-inflammatory drugs (NSAIDs) for 4 weeks, (4) Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) > 4 at baseline, and (5) no TNFi treatment in the 6 months prior to recruitment. The exclusion criteria were as follows: (1) antibiotic or probiotic treatment within 2 months of fecal collection, and (2) chronic infectious disease such as tuberculosis or hepatitis B. Fecal samples and clinical data, including demographic information, disease-related characteristics, and measurements of disease activity or functional status, were collected by trained investigators at baseline (M0) and after 6 months of adalimumab treatment (M6). Disease activity of AS was assessed using the Ankylosing Spondylitis Disease Activity Score (ASDAS) according to its cutoff points, while therapeutic response was defined using the change in ASDAS ($\triangle$ASDAS) as reported previously (14, 15). Clinical response was defined as ASDAS change > 1.1, and no clinical response was defined as ASDAS change ≤ 1.1. In order to determine the suitable sample size, we hypothesized that, after treatment with adalimumab, 50-60% of patients would experience an alteration in the abundance of potentially disease-related components of gut microbiota (16). A total sample size of at least 13 pairs of AS patients (pre- and post-treatment) was required to achieve a power of 90% and a two-sided significance of 5%. The sample size was estimated by PASS 15 software (https://www.ncss.com).

Healthy controls were recruited from volunteers, at the same hospital, who had not been diagnosed with AS, other rheumatic diseases, or chronic infectious diseases, and who had not received antibiotic or probiotic treatment within 2 months of fecal collection.

This study was conducted in compliance with the Declaration of Helsinki and was approved by the ethics committee of the Third Affiliated Hospital of Sun Yat-sen University. Before enrollment, written informed consent was obtained from all subjects for research and publication of their data.

Fecal Sample Collection

Fresh fecal samples were collected from the patients at baseline and at 6 months, and from healthy controls at baseline. After defecation fecal samples were collected and immediately placed on dry ice, and then transferred to the laboratory within 2 hours, followed by the storage at -80°C until DNA extraction.

DNA Extraction and 16S rRNA Gene Sequencing

Microbial genomic DNA was extracted from fecal samples using a DNA isolation kit (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions. The V4 region of the 16S rRNA gene from each sample was amplified by polymerase chain reaction (PCR) using specific primers (515F, 5'-GTGCCAGCMGCCGCGGTAA-3', and 806R, 5'-GGACTACHVGGGTWTCTAAT-3') with barcodes (17). After purification, the DNA library was obtained and sequenced using the Illumina Hiseq2500 platform to generate 250 bp paired-end reads.

The reads were purified and merged and then processed using a QIIME-based bioinformatics pipeline (v1.9.1) (18). Briefly, we curated the sequences to reduce sequencing and PCR errors, aligned the resulting sequences to the SILVA 16S rRNA sequence database, and used UCHIME to remove any chimeric sequences as per to the GOLD database. Sequences were clustered into operational taxonomic units (OTUs) with a 97% similarity cutoff using the average neighbor algorithm. All sequences were classified using a naïve Bayesian classifier trained on the RDP training set, and the OTUs were assigned a classification based on the taxonomy with the majority consensus of sequences within a given OTU at a threshold of 80%. We obtained the OTU table and taxonomy tree, and further analysis of the α- and β-diversity indices was conducted using Microbiomeanalyst (19). It is a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data.

Statistical Analysis

Graphpad 8.0 (IBM, USA) and R (version 3.4.3) were used to analyze the data. Longitudinal comparisons were used to analyze changes in patients after treatment, and cross-sectional comparisons were made between patients and healthy controls.

Gut microbiome composition was represented by α- and β-diversity. To assess α-diversity (20), the Shannon and Simpson

indices were calculated for each sample in the dataset. Wilcoxon rank sum tests were performed for pairwise comparisons within patients with AS, and Mann-Whitney tests were performed for comparisons between patients and healthy controls. To measure β-diversity, the UniFrac distance between samples was calculated (21), and permutational multivariate analysis of variance using distance matrices (PERMANOVA) was used to assess the overlap of taxonomy between patients with AS and healthy controls. Comparisons of the relative abundance of taxa were made using Wilcoxon rank sum or Mann-Whitney tests. A *P* value of less than 0.05, after correcting for false discovery rate (FDR) for multiple comparisons, was considered statistically significant.

RESULTS

Clinical Characteristic of AS Patients

A total of 30 patients (mean age, 31.23 ± 7.48 years) and 24 healthy controls (mean age, 38.54 ± 10.79 years) were obtained in this study. All included patients were HLA-B27 positive, 12 of them (40%) had peripheral arthritis and 5 (17%) had a positive family history. None of these 30 AS patients had established IBD, psoriasis, or uveitis history. Before treatment, 15 patients had a high level of disease activity and 13 patients had very high level of disease activity. NSAIDs and sulfasalazine (SASP) were prescribed for 20 and 5 patients respectively. During adalimumab therapy, NSAIDs and SASP were remained for those patients.

Overall, clinical symptoms and signs of AS patients were greatly relieved after 6 months of adalimumab therapy, along with improvements in C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), BASDAI, ASDAS, Bath Ankylosing Spondylitis Functional Index (BASFI), and Bath Ankylosing Spondylitis Metrology Index (BASMI), comparing with baseline values (all *P* values < 0.01, **Table 1**). Six patients still had high or very high levels of disease activity even after treatment. As for treatment response, 22 patients responded to adalimumab therapy (△ASDAS > 1.1, R) while 8 patients did not (△ASDAS ≤ 1.1, NR). Patients exhibiting different clinical responses were similar in terms of sex, age, and disease activity before treatment (**Table S1**).

TABLE 1 | Clinical characteristic of AS patients.

	M0	M6
Age (years)	31.23 ± 7.48	
Males (n, %)	27 (90)	
Duration (years)[§]	10 (5)	
NSAIDs (n, %)	20 (66.7)	
SASP (n, %)	5 (16.7)	
CRP (mg/L)[§]	11.25 (21.70)	0.95 (4.25)***
ESR (mm/h)[§]	14.00 (25.50)	4.50 (6.25)***
BASDAI[§]	5.23 (1.51)	2.23 (2.72)***
BASFI[§]	3.72 (2.64)	1.60 (2.35)***
BASMI[§]	2.50 (3.00)	1.00 (3.00)**
ASDAS[§]	3.43 (1.29)	1.33 (1.16)***
Inactive[†]	0	14
Low activity[†]	2	10
High activity[†]	15	5
Very high activity[†]	13	1
BASDAI > 4[†]	30	4

*[§]Data expressed as median (IQR); [†]data expressed as frequency. Comparisons of CRP, ESR, BASDAI, BASFI, BASMI, and ASDAS between M0 and M6 were calculated using Wilcoxon rank-sum tests. **P < 0.01, ***P < 0.001. AS, ankylosing spondylitis; IQR, interquartile range; NSAIDs, non-steroidal anti-inflammatory drugs; SASP, sulfasalazine; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; BASFI, Bath Ankylosing Spondylitis Functional Index; BASMI, Bath Ankylosing Spondylitis Metrology Index; ASDAS, ankylosing spondylitis disease activity score; M0, baseline; M6, after 6 months of treatment.*

Association Between Gut Microbiome and Adalimumab Therapy

In the present study, an average of 65 194 (range, 45 295–78 205) high-quality effective reads were obtained from 84 samples. After taxonomic identification and filtering out taxa with a very low abundance (reads had to be present in at least 20% of the samples, with a count of more than 2), 602 OTUs remained for further analysis. After species annotation, a total of 9 phyla, 16 classes, 24 orders, 47 families, and 117 genera were obtained.

For α-diversity, the Shannon and Simpson indices of AS patients before treatment (AS_M0) were obviously lower than healthy controls (*P*<0.01, **Figure 1A**), and there were no statistical differences of the above indices between two groups after treatment (*P*>0.05, **Figure 1A**). Similarly, noteworthy difference in β-diversity between the two groups was found before treatment (*P* < 0.001), while no statistical difference was found after treatment, according to the results of PERMANOVA based on weighted and unweighted UniFrac distance (**Figures 1B, C**). These results revealed an alteration of gut microbial community structure in AS patients and an impact of adalimumab treatment on the gut microbiota.

The three most dominant phyla were *Firmicutes*, *Bacteroidetes*, and *Actinobacteria*, which accounted for over 95% of total abundance both in AS patients and healthy controls. The top fifteen relative abundances of taxa at different taxonomic levels in patients with AS and in healthy controls are listed in **Table S2**.

At the phylum level, gut microbiota from AS patients before treatment exhibited a significantly higher abundance of *Actinobacteria*, *Firmicutes*, *Oxyphotobacteria*, *Preteobacteria*, and *Tenericutes* (all *P* <0.01, **Figure 2**), as well as a lower abundance of *Bacteroidetes* and *Fusobacteria* than the microbiota from healthy controls (*P* <0.001 and *P* <0.05, respectively, **Figure 2**). Interestingly, the relative abundance of these seven phyla shifted during adalimumab therapy, eventually resulting in no statistical difference between the two groups (all *P* >0.05, **Figure 2**). At the genus level, 73 genera with distinguishing abundance (such as *Bacteroides*, *Megamonas*, and *Collinsella*) were identified in AS patients before treatment compared with healthy controls (all *P*<0.05, **Table S3**). Relative abundance of these genera altered during adalimumab therapy, and in particular four genera namely *Lachnoclostridium*, *Lachnospira*, *Solobacterium*, and *Rothia* shifted greatly during this time (all *P*<0.05, **Figure 3** and **Table S3**). The relative abundance of *Lachnoclostridium*, *Lachnospira*, and *Solobacterium* in AS patients was restored to levels similar with healthy controls after therapy (*P*>0.05, **Figure 3** and **Table S3**). Only 28 out of the 73 genera (such as *Rothia, Streptococcus, Blautia*,

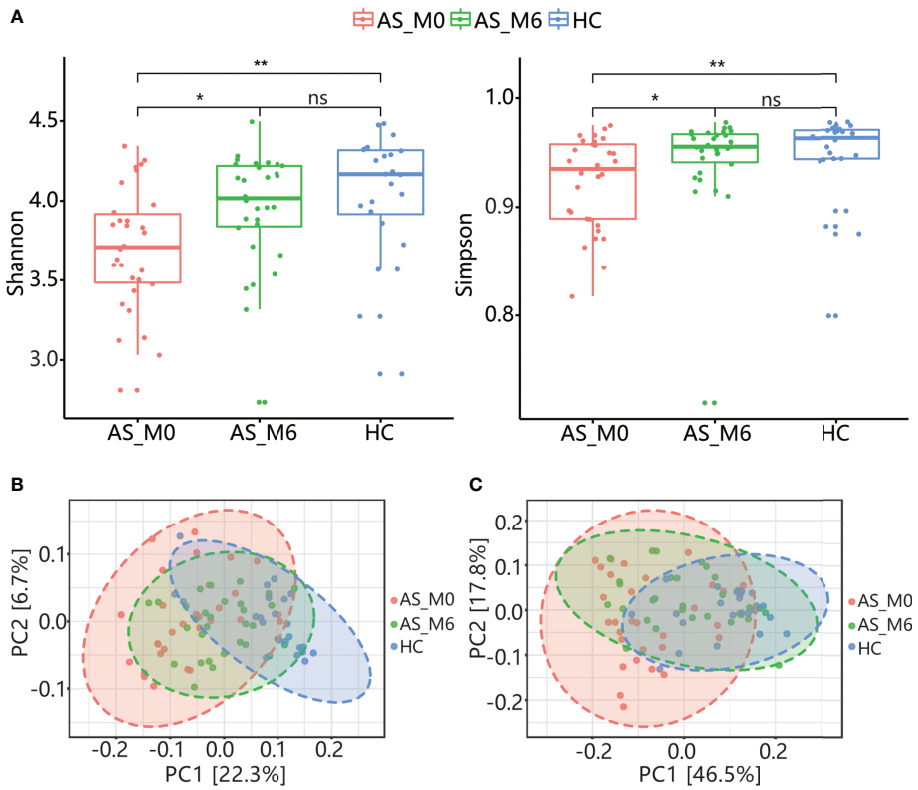

FIGURE 1 | Community structure of gut microbiota in AS patients and HCs. **(A)** α-diversity of gut microbiota (Shannon and Simpson index) among AS patients at baseline and after treatment and among HCs. The horizontal bar within each box represents the median. The bottom and top of each box represent the 25th and 75th percentiles, respectively. The upper and lower whiskers extend to data no more than 1.5 × the IQR from the upper and lower edges of the box, respectively. *P < 0.05, **P < 0.01, ns, P > 0.05. PCoA plot based on the unweighted UniFrac distance **(B)** and weighted UniFrac distance **(C)** of gut microbiota from AS patients at baseline and after treatment and from HCs. AS, ankylosing spondylitis; AS_M0, AS patients at baseline; AS_M6, AS patients after treatment; HC, healthy control; IQR, interquartile range; PCoA, principal coordinates analysis.

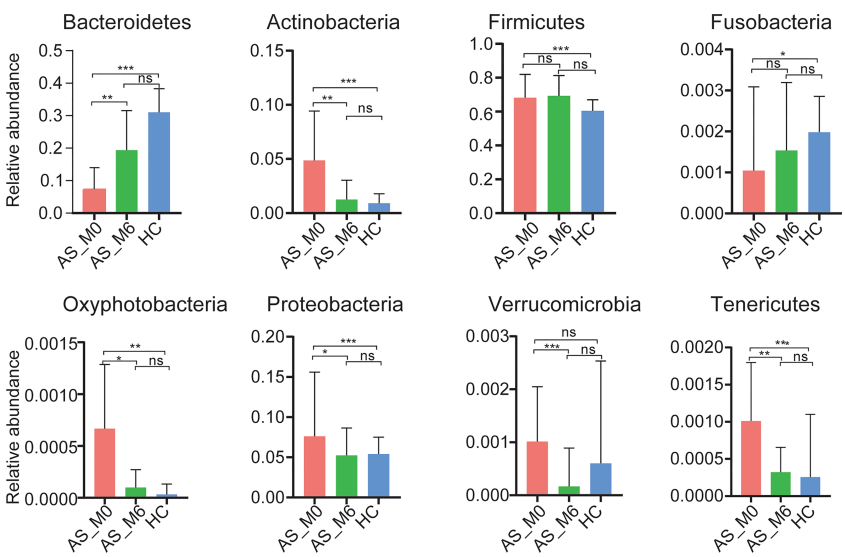

FIGURE 2 | Relative abundance of phyla in different groups. Bars represent the mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ns, P > 0.05.

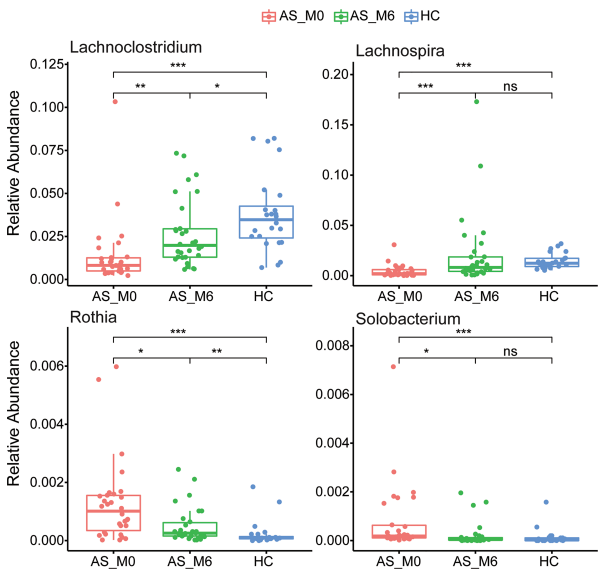

FIGURE 3 | Differential relative abundance of genera in AS patients before and after treatment. AS, ankylosing spondylitis. The horizontal bar within each box represents the median. The bottom and top of each box represent the 25th and 75th percentiles, respectively. The upper and lower whiskers extend to data no more than 1.5 × the IQR from the upper and lower edges of the box, respectively. *P < 0.05, **P < 0.01, ***P < 0.001, ns, P > 0.05.

and *Dorea*) remained statistically different (all P<0.05, **Table S3**) between the two groups after therapy.

Association Between Gut Microbiome and Response to Adalimumab Therapy

We further investigated whether the gut microbial community structure in patients with AS before adalimumab therapy was related to the treatment response. No significant differences of clinical status as well as α- and β-diversity were found between the responders and non-responders (**Table S1** and **Figures 4A–C**). The abundance of microbiota at different taxonomic levels were then analyzed and no differences were found at the phylum, class, order, or family levels, either. At the genus level, a higher abundance of *Comamonas* was observed in the non-responder group (**Figure 4D**). Unfortunately, after adjusting for FDR, no statistical difference was found.

DISCUSSION

Ankylosing spondylitis is a chronic inflammatory disease that is thought to be associated with the gut microbiota. Previous studies have suggested that TNFi therapy improves the gut microbial community. In the current study, we illustrated the effect of adalimumab on the gut microbiota composition in AS patients during 6 months of treatment. We observed that the

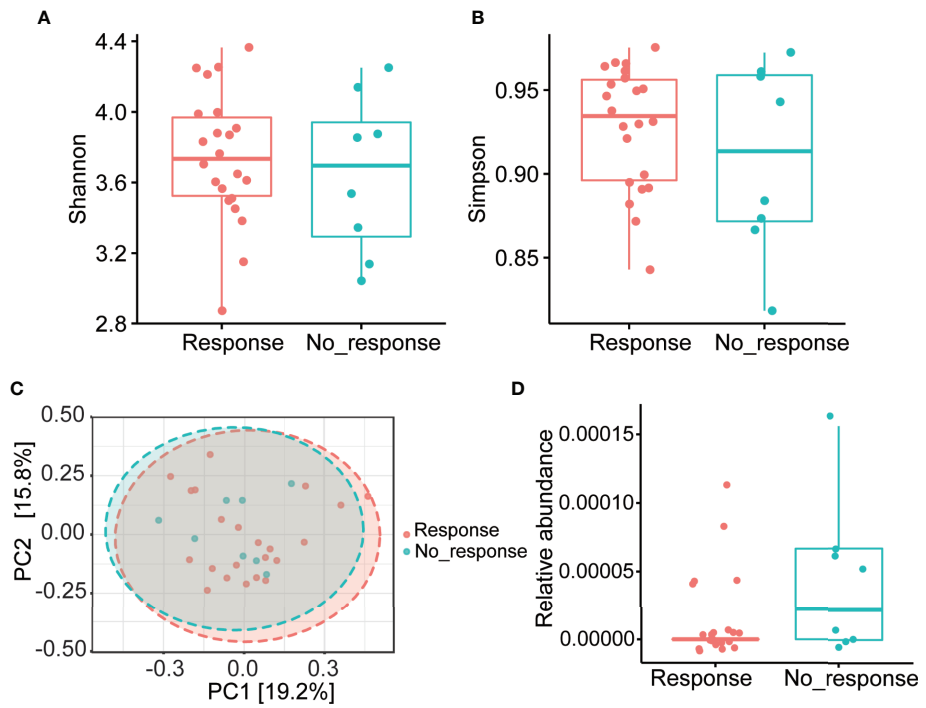

FIGURE 4 | Gut microbial community structure of AS patients before treatment with different responses to Adalimumab. **(A, B)** α-diversity; **(C)** PCoA based on unweighted UniFrac distance. **(D)** Relative abundance of *Comamonas* in response and no_response subgroups. AS, ankylosing spondylitis; PCoA, principal coordinates analysis.

overall gut microbial composition of AS patients exhibited obvious differences comparing with healthy controls, as did most bacterial taxa. After 6 months of adalimumab treatment, the gut microbial composition was restored to a state similar with healthy controls. In addition, no difference was observed in the overall gut composition between the responders and non-responders in AS patients. A higher abundance of *Comamonas* was observed in the non-responders, but this result was not statistically significant after adjustment.

Decreased gut microbiota diversity occurs in many diseases, including IBD and AS. In the current study, we confirmed that gut microbiota diversity was reduced in AS. In addition, we identified bacterial species which were differentiated between AS patients and controls. Depletion of *Bacteroides* and *Megamonas* and enrichment of *Collinsella* in AS patients were noted in our study, which was consistent with findings of previous studies (22, 23). All of these three species restored to indistinguishable from healthy controls after treatment. Carriage of *Dialister* species has been previously reported be associated with disease activity in SpA patients (6). However, we found depletion of *Dialister* in AS patients before treatment and it was restored to similar with healthy controls after treatment. Neither Yin et al. (16) revealed remarkable difference of *Dialister* in AS patients. So, the pathogenic significance of *Dialister* is therefore uncertain.

Restoration of the gut microbiota after treatment has been reported in several other autoimmune diseases, such as IBD and RA (24). One previous study analyzed patients with SpA before and after TNFi therapy and revealed modest differences in microbial composition, but no specific taxon was found to be modulated, which is likely due to the small sample size (8). In another previous study comparing AS patient with and without TNFi treatment, restoration of the overall microbial composition and the abundance of specific taxa were both revealed (16). These indicated TNFi therapy was correlated with a restoration of the perturbed gut microbiota. However, the previous studies did not assess the change in the gut microbiota dynamics of each individual patient. In our study, we compared the baseline state of the gut microbiome of each patient to that 6 months after treatment. Adalimumab therapy was associated with restoration of the microbial composition, and several notable bacterial species modulated by the treatment were identified. We observed that adalimumab therapy restored the normal abundances of *Bacteroidetes* and *Firmicutes*. A decrease in the *Bacteroidetes/Firmicutes* ratio has been associated with autoimmune diseases. Moreover, at the genus level, the abundance of four genera were found to shifted greatly during treatment. Specifically, *Lachnoclostridium* was reduced in AS patients and increased to a level similar with healthy controls after treatment. *Lachnoclostridium* was also found to be less abundant in children with autism spectrum disorders in a previous study (25). As known, AS patients are prone to mental disorders such as depression (26), the effect of perturbations in the gut microbiome on mental disorders in AS patients is worth investigation.

Although great improvement has been reported in some AS patients treated with TNFi, nearly 30% of patients show no response. In the current study, we observed a comparable proportion that 8 out of 30 enrolled patients showed no response to TNFi therapy Thus it is important to identify non-responsive patients, as TNFi therapy is costly. An increasing number of studies have indicated that the gut microbiome was a potential indicator of the response to TNFi treatment, and gut microbiome plays an important role in drug efficacy (27–29). To our knowledge, only one previous study has utilized gut microbiome features to predict the TNFi response in SpA patients in which only 8 non-responders and 5 responders were included (8). A higher abundance of *Burkholderiales* orders was observed in responders prior to treatment. In our study, we included a larger cohort and longer treatment durations and a higher abundance of the *Comamonas* genus in non-responders to adalimumab prior to treatment were revealed. *Comamonas* species are infrequently reported as an infectious agent in routine clinical practice due to rare isolates in microbiology laboratories. In recent years, *Comamonas kerstersii and Comamonas testosteroni* were identified to cause appendicitis and bacteremia by microbiome sequencing (30, 31). Subclinical gut inflammation is common in AS patients, but its relationship with *Comamonas* needs further study. The discrepancy between our results and those of the prior study may be due to differences in racial composition, dietary habits, and treatment duration. These two studies together suggest a possible association between gut microbial features and clinical response, without presuming causality. If the results are further confirmed, it could be clinically helpful to use gut microbiome features as a reliable indicator of treatment response before the initiation of TNFi therapy to avoid a delay in symptom relief and ease the economic burden on health services, paving the way for precision therapy for AS.

Our study had several limitations. Firstly, patients treated with other TNFis or other treatments such as NSAIDs were not recruited, so we cannot conclude that the restoration of the microbiota was specifically related to adalimumab treatment. Secondly, as information of dietary patterns was not collected, the influence of diet was not taken into account. However, since we compared the same patients before and after treatment, any effects of diet should have been somewhat reduced. Finally, due to the small size of our cohort, the power of the statistical analysis may be limited. Nevertheless, in our study, nearly 30% of the patients showed no response to adalimumab therapy, which was consistent with previous larger-scale studies, thereby enhancing the external validity of our study. Further prospective, large-scale studies are required to confirm our results.

CONCLUSION

In summary, our study revealed the gut microbiota features in patients with AS and provided insights into the dynamic alterations during adalimumab treatment. Our investigation suggests that the gut microbiota may be a potential tool for predicting the treatment response to adalimumab in AS patients. Additional studies are needed to further investigate the exact

bacterial species that play key roles in the response to adalimumab treatment, which will be helpful to achieve precise medical interventions.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the SRA repository, accession number is PRJNA755445.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics committee of the Third Affiliated Hospital of Sun Yat-sen University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

(I) Conception and design: JG and ZC. (II) Administrative support: JG. (III) Provision of study materials or patients: All authors. (IV) Collection and assembly of data: ZC. (V) Data analysis and interpretation: All authors. (VI) Manuscript writing and revision: All authors. (VII) Final approval of the manuscript: All authors. (VIII) Funding acquisition: JG. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.700570/full#supplementary-material

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Genetic Associations Between IL-6 and the Development of Autoimmune Arthritis Are Gender-Specific

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Objectives: To find out the genetic association between IL6 and autoimmune arthritis.

Methods: We performed a two-sample Mendelian randomization (MR) study using multiple genome-wide association studies (GWAS) datasets. Furthermore, a sex-stratified MR study was performed to identify sexual dimorphism in the association between IL6 and autoimmune arthritis. Then, LocusZoom plots were displayed based on the IL6R gene region to present evidence of genetic colocalization between diseases.

Results: The MR result denoted a genetic association between the increased level of IL-6 signaling and risk of RA (β=0.325, 95%CI 0.088, 0.561, p=7.08E-03) and AS (β=1.240, 95% CI 0.495, 1.980, p=1.1E-03). Accordingly, sIL6R was found to have negatively correlation with the onset of RA (β=-0.020, 95%CI -0.0320, -0.008, p=1.18E-03) and AS (β=-0.125, 95%CI -0.177, -0.073, p=2.29E-06). However, no genetic association between IL6/sIL6R and PsA was detected. The gender-stratified MR analysis showed that IL6 was associated with AS in the male population, with RA in the female population, and with PsA in the male population. Additionally, ADAR, a gene identified by a sensitive test, could be the reason for the nonsignificant association between IL6 and PsA in a pooled population.

Conclusion: Our findings showed that the overactive IL6 signal pathway led to autoimmune arthritis, especially in RA and AS. Sexual difference was also observed in IL6-intermediate susceptibility to autoimmune arthritis.

Keywords: Mendelian randomization, IL6, sIL6R, autoimmune arthritis, ADAR

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HIGHLIGHTS

1. Genetic association was identified between IL6-signaling and autoimmune arthritis.
2. IL6 was correlated with male AS, male PsA, and female RA development in a sex-dependent manner.

INTRODUCTION

Interleukin-6 (IL-6), as a proinflammatory cytokine, plays major roles in the inflammation process and the development of the immune system (1, 2). However, abnormal immune response could lead to autoimmune arthritis including rheumatoid arthritis (RA), ankylosing spondylitis (AS), and psoriatic arthritis (PsA), which places a great burden on patients by causing joint destruction and even ending up with disability (3, 4). As previously reported, elevating levels of IL-6 have been detected in pathological sites among RA, AS, and PsA patients, and the serum level of IL-6 is associated with the autoimmune arthritis pathogenesis (5–7). Hence, IL-6 blockade seems to be a therapeutic target for alleviating the process of autoimmune arthritis. Anti-IL-6 signaling drugs such as tocilizumab and siltuximab have been approved for RA and systemic juvenile idiopathic arthritis therapy by the Food and Drug Administration (FDA) (4). However, treating AS or PsA with anti-IL-6 therapy could only relatively alleviate the progression of diseases in some reports (8, 9).

It is known that genetic susceptibility accounts for 30% in terms of the risk of autoimmune disease (10). By using information from genome-wide association studies (GWAS), Mendelian randomization (MR), a newly introduced approach, has the potential to evaluate the causal relationship between exposures and phenotype (11, 12). Briefly, MR uses genetic variants as the instrumental variable to interpret a different outcome without bias caused by reverse causation or confounding factors (13, 14). Previous MR studies have shown that the genetic level of serum sIL6R is negatively associated with the risk of developing RA (15). Li and his colleges found that the IL-6 gene -174G/C variant is associated with the risk of RA using MR meta-analysis (16). In order to evaluate the casual association between IL-6 and other autoimmune arthritis, large-scale MR study should be carried out to verify the exact correlation and find out potential therapeutic strategy.

In this study, up-to-date GWAS level summary data for IL-6 signaling and circulating sIL6R were utilized in the MR analysis. The results presented a genetic association between IL6 and different types of autoimmune arthritis. Additionally, we further discovered the underlying sexual dimorphism in these autoimmune diseases based on the MR results.

METHOD

Genetic Instrumental Variables

Two sets of IL6-related genetic instruments were based on recent MR reports. Genetic variants that represented for IL6 signaling were obtained from a large-scale GWAS meta-analysis assessing chronic inflammation, which included 204,402 European individuals (17). Based on prior MR reports from Kappelmann et al. and Georgakis et al. (18, 19), who investigated the association between IL6 signaling on specific depressive symptom outcomes and cardiovascular outcomes, respectively, IL6 signaling-related SNPs were used in our study (**Table S1**). According to Kappelmann et al.'s definition, "IL6 signaling" referred to IL-6R genetic instruments and weighted by the level of CRP.

Plasma sIL6R is a decoy receptor that is able to suppress IL6 signaling by forming an inhibitory complex with sgp130 (20). To further identify the role of IL6 signaling in autoimmune arthritis, genetic IV SNPs associated with the level of sIL6R were generated from a European ancestry GWAS (n=3,301) (21). A total of 34 SNPs utilized in this study were based on Rosa et al.'s study (**Table S1**) (15). Linkage disequilibrium levels of SNPs chosen above from two datasets were tested using the LDmatrix Tool (https://ldlink.nci.nih.gov/?tab=ldmatrix, CEU; r2 < 0.1). When IV SNPs could not be found in autoimmune arthritis summary statistics, the LDproxy Tool was used to identify potential proxy SNPs (CEU; r2>0.8).

Summary Statistics for Autoimmune Arthritis

Publicly available meta-analysis datasets for autoimmune arthritis (RA, AS, PsA) were derived from some different studies. GWAS summary statistics of 14,361 RA cases and 43,923 controls of European ancestry were obtained from a large-scale GWAS (22). The summary of the GWAS statistic for AS and PsA was obtained from FinnGen, a large-scale project combining genotype data from Finnish biobanks and digital health record data from Finnish health registries. We selected datasets containing 541 AS cases, 74,589 controls and 562 PsA cases, and 93,959 controls of Finnish ancestry. Another set of autoimmune arthritis meta-analysis summary statistic was acquired from UK Biobank (UKBB) for replication study, encompassing 361,141 of European ancestry with 4,017 RA cases, 3,154 AS cases, and 712 PsA cases.

Moreover, summary GWAS results of these three types of autoimmune arthritis in different genders were extracted from UKBB (with 194,174 female and 167,020 male) for further sex-stratified MR analysis to avoid potential sexual bias, due to the fact that gender differences had been widely reported in autoimmune diseases.

Statistical Analysis

To evaluate casual effect, an MR study was carried out with four modes: MR-Egger mode, weighted median mode, and inverse variance-weighted (IVW) mode with the "TwoSampleMR" R-package (23). With the minimum weighted average variance, the IVW method was mainly selected for analysis (24). Because the MR analysis is based on the hypothesis that genetic exposure influences the outcome directly, it is not suitable to perform the analysis in the presence of pleiotropy. Thus, we conducted several approaches to detect potential pleiotropy (25), Firstly,

MR-Egger regression was performed to capture the horizontal pleiotropy (26). Additionally, we applied MR pleiotropy residual sum and outlier test (MR-PRESSO) to detect potential horizontal pleiotropy using the MR-PRESSO global test. For any detected pleiotropic SNP, the MR-PRESSO outlier test was performed to remove these SNPs and rectify the horizontal pleiotropy (27).

Heterogeneity test was performed using Cochran's Q statistics analysis to identify whether the MR results were biased by the heterogenic factors. Leave-one-out sensitivity analysis was performed to identify whether the estimate was driven by an SNP. These two methods were also applied through the "TwoSampleMR" R-package. The estimated effect represented the log odds ratio (OR) between IL6R and autoimmune arthritis risk. All the analyses with P<0.05 were considered statistically significant.

RESULTS

Potential genetic association between the levels of IL6-signaling, sIL6R, and autoimmune arthritis were detected by using two-sample MR analysis (**Figure 1**). Summarized results of associations are shown in **Table 1**, and a forest plot is shown in **Figure 2**.

Finding for Upregulated IL6-Signaling and Levels of sIL6R in Autoimmune Arthritis

Casual effects of upregulated IL6-signaling have been found to be positively associated with RA (IVW mode effect=0.325, 95%CI 0.088 to 0.561, p=7.08E-03) and AS (IVW mode effect=1.240, 95%CI 0.495 to 1.980, p=1.1E-03). As the results of MR present, the elevated concentration of sIL6R, which inhibits IL6-signaling, was negatively associated with the risk of RA (IVW mode effect=-0.020, 95%CI -0.0320 to -0.008, p=1.18E-03) and AS (IVW mode effect=-0.125, 95%CI -0.177 to -0.073, p=2.29E-06). Replicated MR analysis confirmed the connection between IL6 and risk of RA (IVW mode effect=0.003, 95%CI 0.007 to 0.011, p=1.98E-04) or AS (IVW mode effect=0.0039, 95%CI 0.0019 to 0.0059, p=1.51E-04). The negative association between sIL6R and AS was validated through replicated MR analysis (IVW mode effect=-0.00033, 95%CI -0.00048 to -0.00018, p=-0.00018). However, no genetic association was found between RA and sIL6R in the replicated MR analysis. Similar with the MR analysis results in PsA, no genetic effects were found between the risk of PsA and IL6-signaling (**Table 1**).

Pleiotropy test using MR-Egger did not denote any pleiotropic SNP in the MR study. However, the horizontal pleiotropy was detected among the MR results by using MRPRESSO (**Table 1**). After removing the pleiotropic SNPs by the MRPRESSO outlier test, the corrected MR estimates are listed in **Table S2**.

Furthermore, we carried out Cochran's Q statistics test to evaluate the MR results. There was no sign of heterogenetic effects between the genetic effects and risk of AS (**Table 1**). However, heterogeneity was found among the MR estimate concerning the association between IL6 and two other autoimmune arthritis diseases (**Table 1**). Thus, leave-one-out sensitivity analysis was performed to identify the important SNP and the possible source of heterogeneity (**Figure S1**). Rs2228145, a well-reported IL6 receptor genetic variant, was confirmed to strongly influence the effects of IL6-signaling on RA or AS (28) (**Figure S1**). In addition, rs4129267, an IL6R-related SNP in LD with rs2228145(r2 = 1) identified by Ferreira and her colleges (29), was found to make a great contribution to getting RA. On the other hand, rs12059682 had a biased role of IL6 in the onset of PsA (**Figure S1**).

Gender-Stratified MR Analysis for IL6-Signaling and Levels of sIL6R in Autoimmune Arthritis

Sexual dimorphic effects on autoimmune arthritis were widely demonstrated previously. In order to further identify the potential heterogeneity in order to further identify among MR analyses. We performed sex-stratified MR analysis to judge whether a sex-specific genetic relationship existed between the

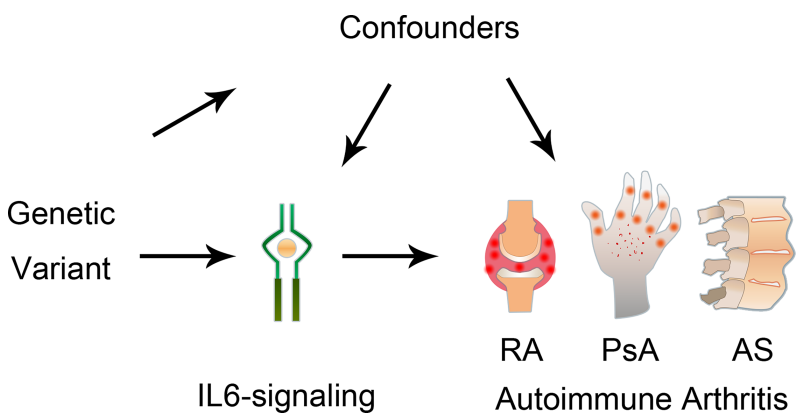

FIGURE 1 | Graphical diagram of the Mendelian randomization analysis between IL6 and risk of autoimmune arthritis. RA, rheumatoid arthritis; AS, ankylosing spondylitis; PsA, psoriatic arthritis.

TABLE 1 | MR estimates of the causal effect of IL6-signaling and sIL6R level on the risk of autoimmune arthritis.

Exposure	MR method	Outcome	Association			Heterogeneity		Egger regression	MR PRESSO
			beta	se	pval	Q	P-value	P-value	P-value
IL6-signaling	ME	RA	0.053156	0.46213	0.914	19.34	**0.0017**	0.5733	**0.015**
	WM	(Euro)	0.327559	0.07645	**2E-05**				
	IVW		0.324962	0.12067	**0.0071**				
	ME	RA	-0.00496	0.0071	0.5234	3.54	0.6174	0.1430	0.09
	WM	(UKBB)	0.005779	0.00241	**0.0163**				
	IVW		0.007435	0.002	**0.0002**				
	ME	AS	1.470001	1.53846	0.3934	5.33	0.3766	0.8838	**0.021**
	WM	(Finngen)	1.434537	0.46354	**0.002**				
	IVW		1.239619	0.37985	**0.0011**				
	ME	AS	0.003159	0.00363	0.433	1.30	0.9352	0.8485	0.237
	WM	(UKBB)	0.003863	0.00122	**0.0015**				
	IVW		0.003868	0.00102	**0.0002**				
	ME	PsA	1.20307	2.09803	0.5971	11.21	**0.0474**	0.7250	0.069
	WM	(Finngen)	0.579845	0.43903	0.1866				
	IVW		0.441713	0.52576	0.4008				
	ME	PsA	0.002501	0.00548	0.6717	14.02	**0.0155**	0.6613	**0.006**
	WM	(UKBB)	0.000954	0.00108	0.3762				
	IVW		1.77E-05	0.00142	0.99				
sIL6R	ME	RA	-0.02508	0.01238	0.0524	50.80	**0.0074**	0.6351	**0.003**
	WM	(Euro)*	-0.02599	0.0064	**5E-05**				
	IVW		-0.01995	0.00615	**0.0012**				
	ME	RA	-0.00021	0.00036	0.5684	48.29	**0.0418**	0.8518	**0.026**
	WM	(UKBB)	-0.00034	0.00021	0.1018				
	IVW		-0.00026	0.00017	0.1283				
	ME	AS	-0.15921	0.05436	**0.0062**	20.85	0.9503	0.4793	**0.035**
	WM	(Finngen)	-0.12799	0.0384	**0.0009**				
	IVW		-0.12524	0.0265	**2E-06**				
	ME	AS	-0.00029	0.00016	0.0697	35.73	0.3414	0.8073	0.085
	WM	(UKBB)	-0.00027	0.0001	**0.0111**				
	IVW		-0.00033	7.6E-05	**2E-05**				
	ME	PsA	-0.07011	0.05904	0.2437	41.86	0.1386	0.5380	0.086
	WM	(Finngen)	-0.04221	0.03518	0.2302				
	IVW		-0.03802	0.02852	0.1825				
	ME	PsA	0.000118	0.00017	0.4816	62.73	**0.0014**	0.1337	**<0.001**
	WM	(UKBB)	-0.00011	8.9E-05	0.2077				
	IVW		-0.0001	8.4E-05	0.2143				

*6 IV SNPs were used in IL6-signaling; 34 IV SNPs were used in sIL6R MR analysis. *30 IV SNPs were identified in the Euro RA database. Significant results are in bold. MR, Mendelian randomization; ME, MR-Egger; WM, weighted median; IVW: inverse variance weighted.*

IL6 and the risk of autoimmune arthritis (**Figure 3**). The UKBB GWAS datasets were used, which include publicly available sex-specific summary data. We found casual association between IL6 and RA in women rather than in men. Conversely, the male population seemed easier to suffer from AS when exposed to elevating IL6-signaling levels (**Table 2**). Consistent with the casual estimate in AS, men could be at higher risk of getting PsA than women. However, it is intriguing that upregulation of IL6-signaling even negatively associated with the risk of PsA (**Table 2**). Next, as shown by MRPRESSO, the pleiotropy in the MR estimate was found only in PsA. After removing pleiotropic SNP from MR analysis, there was no casual association between IL6 and PSA in the female population (corrected estimate listed in **Table S2**).

Next, we apply the leave-one-out sensitive test to evaluate whether some potential SNP creates bias on the gender effects on the MR estimate. No SNP was discovered to create bias in the genetic effect between IL-6 exposure and RA or AS (**Table 2**). Accordingly, Rs2228145 was also verified as the key SNP in the development of PsA in men (**Figure S2**). Because rs12059682 was identified as the potential bias SNP among overall PsA populations, we found that it posed gender difference in the effect contribution to the onset of PsA (**Figure S2**). When leaving it out, upregulating IL6-signaling was also found to be not associated with the risk of PsA.

To deeply identify the role of rs12059682 in the PsA among different genders, firstly, the genetic variant was mapped as the variation of ADAR (adenosine deaminase acting on RNA). Single-tissue eQTL evaluation was carried out using GTEX. Rs12059682 T>C variation would lead to a higher transcriptional level of ADAR in the adrenal gland, EBV-transformed lymphocytes, lung esophagus mucosa, and fibroblast (**Figure S3** and **Table S3**). Based on Shallev et al.'s work (30), decreased A-to-I RNA editing could lead to the accumulation of (double-stranded RNA) dsRNA, which played an important role in psoriasis pathogenesis. Variation of rs12059682 was found riskier in women. Therefore, we brought out the hypothesis that a lower level of ADAR promoted the development of PsA in females.

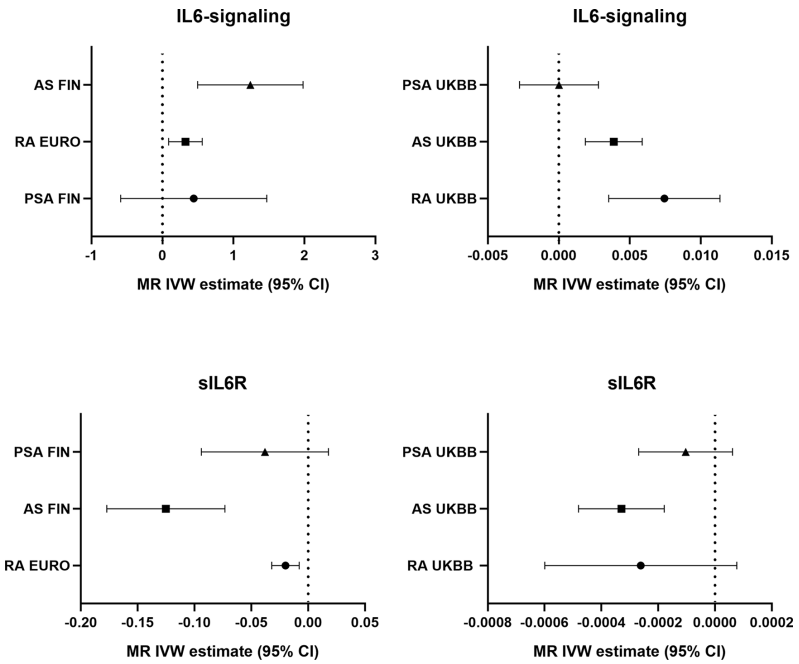

FIGURE 2 | Forest plot of the causal effects of IL6-signaling and sIL6R on autoimmune arthritis.

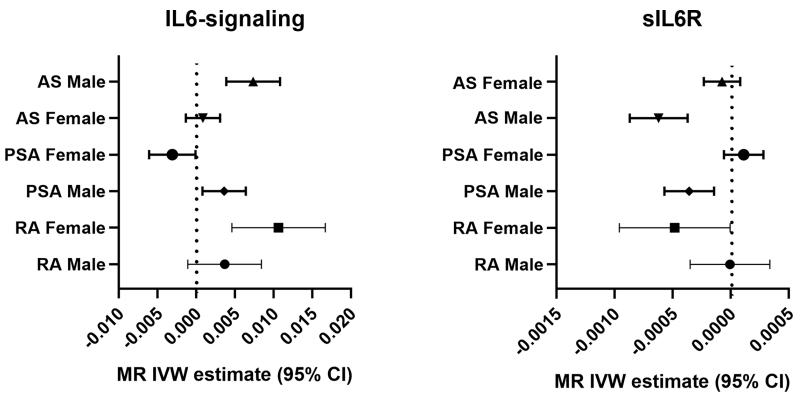

FIGURE 3 | Forest plot of the causal association between IL6-signaling/sIL6R and autoimmune arthritis by sex.

Thus, we browsed the GEO database for sex-stratified PsA gene expression profile. As shown by **Figure 4A**, the transcriptional level of ADAR was lower in female PsA patients than healthy donors (GSE61281, p=0.0371) (31), while there was no statistically significant difference between male PsA patients and the control group (p=0.3333). In addition, the ADAR level was found to be correlated with RA (32). The rs12059682 T>C mutation could result in a higher level of ADAR and increased the risk of developing RA based on the GWAS datasets. Moreover, the level of ADAR was found to be higher in female RA patients compared to age-matched male patients (**Figure 4B**, GSE74143) (32).

Regional Visualization of Autoimmune Arthritis GWAS Datasets for Further Analysis

As shown in **Figure 5**, LocusZoom plots were displayed based on the IL6R gene region (1q21.3) to present evidence of genetic colocalization between diseases. However, no genome-wide level association signals were found in this region. At first, we browsed the IL6R gene region to identify the top SNPs with a p-value lower than 0.01 (MAF >0.01). If the candidate SNP could not be annotated to the RS number and substituted by proxy SNP, then the SNP in this region with the next lowest p-value was chosen for analysis.

TABLE 2 | Gender-stratified MR estimates of the causal effect of IL6-signaling and sIL6R level on the risk of autoimmune arthritis.

Exposure	MR method	Outcome	Association			Heterogeneity		Egger regression	MR PRESSO
			beta	se	pval	Q	P-value	P-value	P-value
IL6	ME	RA	-0.00517	0.010933	0.661162	3.002	0.6996	0.206	0.124
-signaling	WM	(Female)	0.008674	0.003873	**0.02512**				
	IVW		0.010639	0.003077	**0.00054**				
	ME	RA	-0.00465	0.008614	0.617867	2.240	0.8150	0.371	0.669
	WM	(Male)	0.00236	0.002809	0.400914				
	IVW		0.003676	0.002425	0.129564				
	ME	AS	-0.00083	0.004016	0.846193	1.855	0.8689	0.682	0.854
	WM	(Female)	0.000379	0.001309	0.771996				
	IVW		0.000869	0.00113	0.441816				
	ME	AS	0.007835	0.006303	0.28172	0.661	0.9850	0.942	0.105
	WM	(Male)	0.007138	0.002134	**0.00082**				
	IVW		0.007363	0.001774	**3.3E-05**				
	ME	PsA	0.002284	0.005417	0.694948	9.320	0.0970	0.360	**0.046**
	WM	(Female)	-0.00168	0.001378	0.222713				
	IVW		-0.00308	0.001534	**0.04443**				
	ME	PsA	0.002806	0.005655	0.64576	6.215	0.2859	0.888	**0.033**
	WM	(Male)	0.003881	0.001518	**0.01056**				
	IVW		0.003621	0.001428	**0.01122**				
sIL6R	ME	RA	-0.00034	0.000501	0.505225	40.382	0.1764	0.740	0.141
	WM	(Female)	-0.00059	0.000318	0.06377				
	IVW		-0.00048	0.000244	**0.04763**				
	ME	RA	-5E-05	0.000352	0.887085	30.849	0.5746	0.882	0.561
	WM	(Male)	-1E-04	0.000245	0.684076				
	IVW		-4.5E-06	0.000174	0.9794				
	ME	AS	-4E-05	0.000164	0.807609	29.348	0.6496	0.807	0.627
	WM	(Female)	-8.9E-06	0.00012	0.940617				
	IVW		-7.6E-05	8.11E-05	0.351547				
	ME	AS	-0.00059	0.000258	**0.02848**	26.696	0.7728	0.896	0.311
	WM	(Male)	-0.00055	0.000186	**0.00307**				
	IVW		-0.00062	0.000127	**1.1E-06**				
	ME	PsA	0.000286	0.000175	0.111997	38.330	0.2404	0.261	0.319
	WM	(Female)	0.00013	0.000112	0.247094				
	IVW		0.000112	8.7E-05	0.197332				
	ME	PsA	-7.9E-05	0.000218	0.720009	46.942	0.0547	0.154	**0.001**
	WM	(Male)	-0.00032	0.000134	**0.01541**				
	IVW		-0.00036	0.00011	**0.00117**				

Significant results are in bold. MR, Mendelian randomization; ME, MR-Egger; WM, weighted median; IVW, inverse variance weighted

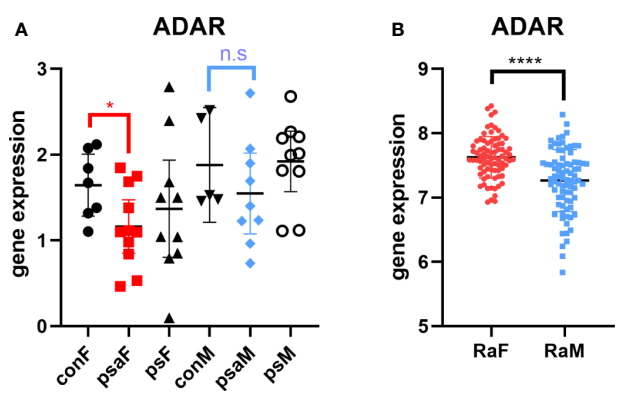

FIGURE 4 | Expression of ADAR in RA or PsA from GEO datasets GSE61281 **(A)** and GSE74143 **(B)**. con, control; psa, psoriatic arthritis; ps, psoriasis; RA, rheumatoid arthritis; F, female; M, male. Data from all bar graphs are presented as mean ± SEM. (*P < 0.05, ****P < 0.0001, n.s, not significant).

By using the Finngen GWAS dataset for AS, rs4845372 (1:154442920_C/A, P=2.48 × 10^-3) was identified as the potential SNP accounting for IL6's effects in the onset of AS. After LD evaluation, this genetic variance was found to be in high linkage with rs2228145 (r2 = 0.882). Next, a sex-stratified plot using UKBB GWAS data presented rs6695045 (1:154432957_A/G, P=1.47 × 10^-3) as the top influencer in the IL6R region with the male population, which was also an SNP that was relatively in linkage with rs2228145 (r2 = 0.438). On the other hand, in the female population, none of the candidate SNPs (p<0.01) was detected in the IL6R gene region. When looking for other than the IL6R gene region, there were no other signals having eQTL data or a literature-based relationship with IL6R.

In addition, our regional plot further emphasized rs2228145 (1:154426970_A/C, P=4.50 × 10^-6) as the lead SNP, putting great genetic predisposition to RA (**Figure 5**). As for further sex-specific analysis, no SNP in the IL6R region met our criteria when analyzing male GWAS data. Rather, a weak signal (1:154410686_C/CT p=0.012) was identified in women with

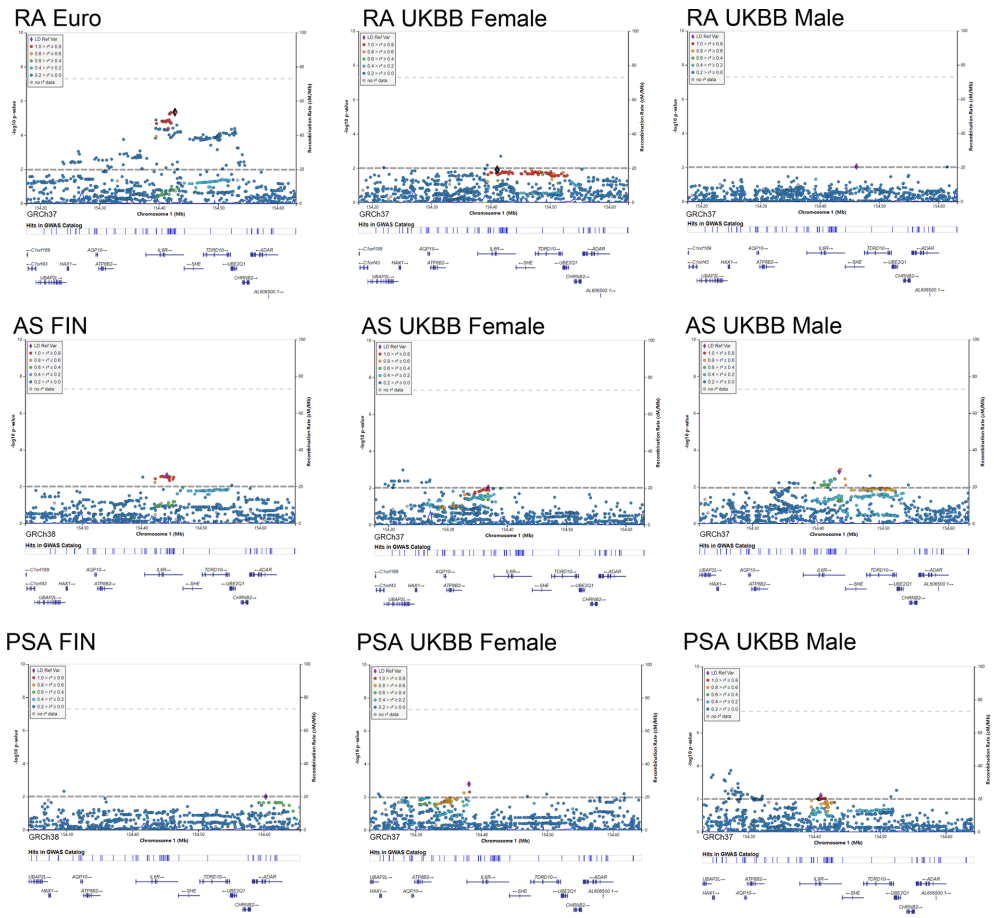

FIGURE 5 | Regional plot of the genetic variants at 1q21.3 and their association with autoimmune arthritis.

RA. The C to T variance of proxy SNP rs55800510 (R2 = 0.9666) was associated with the risk of RA (**Table S3**).

As for the regional plotting result in male PsA patients, we found that rs12730036 (1:154416969_C/T p=9.37 × 10^-3) was in high linkage with rs2228145 (r2 = 0.941). This result denoted consistency with the MR analysis, which resulted in a higher risk of PsA in male by upregulating IL6 signaling. Interestingly, the lowest-p value-polymorphism rs12083537 G>A, which was able to activate the IL6-related pathway, presented to be a protective genetic variance for women against PsA (**Table S3**).

DISCUSSION

In the present MR study, by applying two sets of genetic IVs, our findings showed corroborating evidence that the overactive IL6 signal pathway led to autoimmune arthritis, especially in RA and AS. Genetic predisposition to a higher IL6 level was also genetically associated with the risk of PsA in the male population. Consistent with a previous study, elevating level of IL6 was found in patients with autoimmune arthritis, which played a great role in the pathogenesis of joint lesions (6, 7, 33). As Rosa and her colleague presented, by using sIL6R genetical IV and PheWAS analysis, the genetically determined sIL6R was negatively associated with the risk of RA (15). We extended the result and complemented the evidence from a regional plot and MR analysis. rs2228145, a genetic variant that accounted for 51% variance of sIL6R levels (34), was identified as the common susceptibility locus for autoimmune arthritis. Additionally, the strategy of targeting the IL6 axis by using novel biological medicine to treat RA had been well validated (35). However, anti-IL6 therapy against AS or PsA was still not introduced clinically.

We observed that sexual differences influenced IL6-intermediate susceptibility to autoimmune arthritis. Sex differences were reported in the level of IL-6 among monocytes (36). Consistently, gender differences in the prevalence of RA were well described based on some large epidemiological studies (37). In the previous study, hormonal factors and sex chromosomes were thought to cause the sex difference in autoimmune disease (38, 39). Here, we present the evidence that IL6 acted differently during the onset of RA, which could be another possible explanation for gender difference in RA (40). Likewise, AS is another typical autoimmune arthritis where the

sex ratio is 3:1 (M/F) (41). Our MR analysis confirmed the casual association between IL6 and the development of AS in a gender-dependent manner. However, Gracey and his colleagues found that there was no sex dimorphism in the level of IL-6 among AS patients (42). Additionally, another observational study identified a higher level of IL6 in female patients with syndesmophytes than those without them. No significant change of the IL6 level was found in the pooled populations or male patients with syndesmophytes (43). Here, by using the summary dependent t-test, we found that the IL6 level was higher in male than in female patients without syndesmophytes (3.46 ± 2.86 *vs* 1.47 ± 1.1) (p=0.024). We presumed that the result of the IL6 level was confounded by the progression of diseases. However, the underlying mechanism is still unclear and further study is needed.

As for the onset of PSA, unlike the equal gender ratio observed in epidemiology, we found that a genetically elevating level of IL6 could increase the risk of PsA for men and decrease the risk for women. A cross-sectional analysis reported by Eder et al., who specifically compared the sexual difference in PsA, presented the phenotype that male PsA patients developed more severe axial and joint damage than female (44). Contrarily, some researchers reported that female patients were suffering from RA with more severe joint damages (45, 46). Here we identified a genetic variant rs12059682, located in the ADAR1 gene region, causing heterogeneity in MR analysis. A recent study introduced the role of the IL6R-STAT3-ADAR1 axis in the oncogenicity of multiple myeloma (47). It should be noted that enrichment of ADAR SNP loci was identified in GWAS signals for autoimmune diseases (48). Corresponding to the evidence from the rna-seq dataset, rs12059682 C>T mutation could lead to a lower level of ADAR in the lymphocyte and fibroblast. The decreased level of DNA editing, regulated by the ADAR (49), was correlated with a higher risk of PsA due to the accumulation of dsRNA (30). Furthermore, the impaired post-translational modification of dsRNA and unwinding of dsRNA structures led to adaptive immune activation through producing IFNβ (50). On the other hand, Vlachogiannis et al. observed elevated adenosine-to-inosine RNA editing in the progress of RA (32). Consistently, a higher transcriptional level of ADAR was observed in female RA patients. As Vlachogiannis et al. suggested, more single-stranded RNA (ssRNA) was produced by adenosine-to-inosine RNA editing, which resulted in the binding of ssRNA-binding proteins and increase in the expression of these pro-inflammatory genes (such as HuR). Therefore, the opposite effect of ADAR could be the reason for a different role of IL6 in these two autoimmune arthritis diseases. Elsewise, this SNP could partially account for the sexual difference in RA or PsA susceptibility. However, further sex-stratified GWAS research and pathological studies should be carried out to validate these hypotheses and undermine the potential mechanism.

This MR study was performed by using two sets of IL6-related IV and multiple GWAS datasets, which aimed to ensure the consistency of the results. However, there were still some limitations. Firstly, the sex-stratified MR analysis was mainly based on the UKBB GWAS data. Some genetic variants were different from Finland and UK populations, which was caused by "population bottlenecks" (51). Gender-stratified GWAS datasets from Finland should be collected to replicate the findings, although the allele frequency of major SNP was similar between the two ethnic groups. Moreover, our study identified rs12059682 as the potential source of bias. Other GWAS focused on the level of ADRA editing that should be performed to identify the casual association between ADRA and autoimmune arthritis. Finally, however, the genetic pleiotropy was rectified by the MRPRESSO in the pooled or sex-stratified MR under a sensitive test. Other potential pathway for the associations cannot be fully ruled out.

To summarize, we identified the genetic association between IL6-signaling and onset of autoimmune arthritis. These results further validated IL6 inhibition as the therapeutic strategies for RA. Rather, the genetic role of IL6 in a sex-dependent manner was discovered in the development of male AS, male PsA, and female RA. Those who have started randomized controlled trials of IL6 inhibition on autoimmune arthritis should take gender differences into account. In the end, the hypothesis that ADAR reduces the effectiveness of the IL6 inhibitor in PsA populations, supported by the statistical findings, should be further investigated through pathophysiological studies.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

SY, JH, and WW designed this study. Study conduct: JH and WW. Data collection: ZQ, CL, XJ, and JW. Data analysis: CF, YZ, XJ, and GZ. Drafting the manuscript: JH, ZQ, and XJ. Revising the manuscript content: JM, CJ, YS, and CZ. Approving the final version of the manuscript: all authors. SY takes responsibility for the integrity of the data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.707617/full#supplementary-material

Supplementary Figure S1 | Forest plots and "leave-one-out" plot for MR analyses of the causal effect of IL6 on autoimmune arthritis in pooled population.

Supplementary Figure S2 | Forest plots and "leave-one-out" plot for MR analyses of the causal effect of IL6 on autoimmune arthritis in female or male population.

Supplementary Figure S3 | Violin plot and multiple tissues expression level of rs12059682.

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REVIEW
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Targeting the Interleukin-23/ Interleukin-17 Inflammatory Pathway: Successes and Failures in the Treatment of Axial Spondyloarthritis

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The IL-23/IL-17 pathway has been implicated in the etiopathogenesis of axial spondyloarthritis through studies of genetic polymorphisms associated with disease, an animal model with over-expression of IL-23 that resembles human disease, and observations that cytokines in this pathway can be found at the site of disease in both humans and animal models. However, the most direct evidence has emerged from clinical trials of agents targeting cytokines in this pathway. Monoclonal antibodies targeting IL-17A have been shown to ameliorate signs and symptoms, as well as MRI inflammation in the spine and sacroiliac joints, in patients with radiographic and non-radiographic axial spondyloarthritis. This was evident in patients refractory to non-steroidal anti-inflammatory agents as well as patients failing treatment with tumor necrosis factor inhibitor therapies. Treatment with a bispecific antibody targeting both IL-17A and IL-17F was also effective in a phase II study. Post-hoc analyses have even suggested a potential disease-modifying effect in reducing development of spinal ankylosis. However, benefits for extra-articular manifestations were limited to psoriasis and did not extend to colitis and uveitis. Conversely, trials of therapies targeting IL-23 did not demonstrate any significant impact on signs, symptoms, and MRI inflammation in axial spondyloarthritis. These developments coincide with recent observations that expression of these cytokines is evident in many different cell types with roles in innate as well as adaptive immunity. Moreover, evidence has emerged for the existence of both IL-23-dependent and IL-23-independent pathways regulating expression of IL-17, potentially associated with different roles in intestinal and axial skeletal inflammation.

Keywords: axial spondylarthritis, treatment, IL-23/IL-17 axis, inflammation, disease progression

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INTRODUCTION

Axial spondyloarthritis (axSpA) is an inflammatory disease of the sacroiliac joints (SIJ) and spine that may also involve peripheral joints and entheses (1). It is associated with extra-articular sites of inflammation manifesting as anterior uveitis, aortitis, colitis, and psoriasis. A pathological hallmark is the development of new bone formation as a tissue response to inflammation which is primarily

evident in the SIJ and spine. This may lead to spinal ankylosis, and its severity determines the degree of functional impairment. Disease onset is typically in the third and fourth decades of life and the disease pursues a severe course in about 30-40% of cases, with considerably impairment of quality of life and even increased mortality (2). There is a strong genetic component to its etiology along with alterations in gut microbiome and mechanical factors though the precise steps remain unclear (3). Therapeutics that effectively control inflammation have recently emerged, although these have been identified more by randomized placebo-controlled trials (RCTs) than by studies of basic pathogenesis. Therapeutic agents that target tumor necrosis factor (TNF) have now been used to successfully treat not only joint and entheseal inflammation but also all extra-articular manifestations of disease (4, 5). It remains unclear, however, whether they impact the development of new bone, and about 30-40% of patients fail to attain a sufficient response. Recent attention has pivoted to the interleukin-23(IL-23)/interleukin-17 (IL-17) pathway as data has emerged from animal models and human tissue samples that these cytokines are present at the site of disease and are key effectors of tissue inflammation. However, the picture that has emerged is a complex one as it has become apparent that targeting this pathway is effective in some tissue locations and not others. Our aims in this review were to discuss recent studies investigating the role of the IL-23/IL-17 pathway in the pathophysiology of inflammation and new bone formation in axSpA, data from recent RCTs and other studies informing the tissue specificity of IL-23 and IL-17 targeted therapies, and the new implications of the findings of RCTs for our understanding of the pathophysiology of axSpA.

CLASSIC IL-23/IL-17 PATHWAY

IL-23 is a pro-inflammatory cytokine with a critical role in mediating autoimmunity (**Figure 1**). It promotes the development of a group of cells that express transcriptional factor retinoid-acid-receptor-related orphan receptor γ (RORγt), including T_H17 cells differentiated from naïve T cells, and other IL-17 expressing cells in the innate immune system or non-lymphoid tissues. These RORγt+ cells express a unique panel of inflammatory cytokines and chemokines, including IL-17, IL-22 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (6, 7). The recognition of IL-23 and downstream T_H17 cells challenged the long-standing paradigm of T_H1- T_H2 cells, adding T_H17 cells in the spectrum of immune response (8).

IL-23 is a heterodimer with two subunits, the p40 subunit, shared with IL-12, and the p19 subunit, unique to IL-23. It is primarily secreted by macrophages and dendritic cells in tissues like skin, intestinal mucosa, lungs, synovium, and brain, and signals through the IL-23 receptor (IL-23R) complex. The IL-23R complex consists of two subunits, one is IL23R, and the other is IL-12 receptor (IL-12R) β chain (9), shared with IL-12R. IL-23/IL-23R signaling plays an important role in $T_H17$cell differentiation, survival, and expansion. The initial stage of

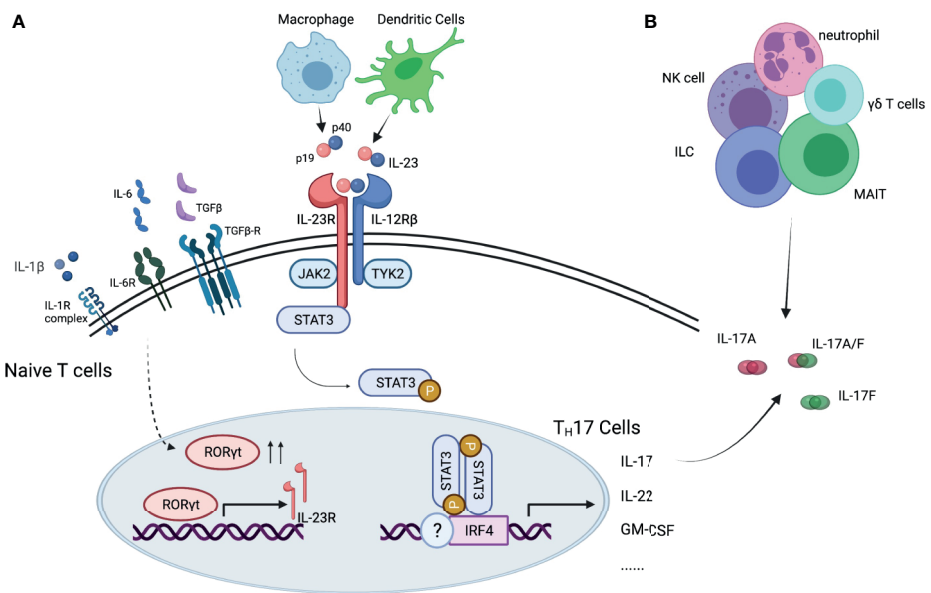

FIGURE 1 | Interleukin-23/interleukin-17 pathway. **(A)** IL-23 is essential for differentiation of T_H17 cells and production of IL-17 cytokine. **(B)** IL-17 cytokine can be produced by other immune cells. IL, interleukin; IL-23R, interleukin-23 receptor; IL-12Rβ, interleukin-12 receptor beta; IL-6R, interleukin-6 receptor; TGFβ, transforming growth factor beta; TGFβ-R, transforming growth factor beta receptor; IL-1R, interleukin-1 receptor; JAK2, Janus kinase 2; TYK2, tyrosine kinase 2; RORγt, retinoid-related orphan receptor gamma t; receptor- STAT3, signal transducer and activator of transcription 3; IRF4, interferon regulatory factor 4; NK cell, natural killer cell; ILC, innate lymphoid cell; MAIT, mucosal associated invariant T cells.

T_H17 cell differentiation from naïve T cells is triggered by IL-6, transforming growth factor β (TGFβ), and IL-1β. The presence of these cytokines promotes the expression of RORγt, signal transducer and activator of transcription 3 (STAT3) and interferon-regulatory factor 4 (IRF4). After the initial trigger, IL-23 is essential for maturation of T_H17 cells, by reinforcing RORγt expression and suppresses differentiation to T_H1, T_H2 or regulatory T cells (Treg) (10), as well as for maintenance of T_H17 signature gene expression, including IL-17, IL-22 and GM-CSF (11).

IL-17 is a family of pro-inflammatory cytokines primarily secreted by T_H17 cells, and by natural killer cells (NK cells), innate lymphoid cells (ILCs), γδ T cells, mucosal-associated invariant T (MAIT) cells. It has six members, from IL-17A to IL-17F, IL-17A being the best described among them (**Figure 2**) (11, 12). It binds to IL-17 receptors, a family of receptors that consist of 5 homologous subunits, IL-17RA to IL17-RE, that are widely expressed in all type of cells. Among them, IL-17RA is the most well studied, primarily expressed in myeloid cells, while IL-17RC is mainly expressed in non-hematopoietic cells, such as epithelial cells, mesenchymal cells and fibroblasts. IL-17 induces expression of pro-inflammatory cytokines (e.g. IL-6 and IL-8) and chemokines that promote neutrophil chemotaxis and accumulation, antimicrobial peptides that protect skin and mucosal surfaces, and molecules that are involved in bone remodeling [e.g. matrix metalloproteinases (MMPs) and receptor activator for nuclear factor-κB ligand (RANKL)] (13, 14).

IL-17A and IL-17F are typically co-expressed by type 17 cells and exist as both homodimers and as a heterodimer. All forms bind to a heterodimeric IL-17RA/IL-17RC complex and trigger qualitatively similar signaling pathways. Both cytokines are increased in a variety of inflamed human tissues and there is synergistic activity with other proinflammatory cytokines, such as TNF, in driving inflammatory pathways (15–17). In a proof-of-concept study of skin and synovial tissue from patients with psoriatic arthritis (PsA), both IL-17A and IL-17F were present in skin lesions and inflamed synovial tissue (18). Moreover, IL-17A or IL-17F did not activate PsA synoviocytes when administered alone, but both cytokines stimulated PsA synoviocytes to produce pro-inflammatory cytokines such as IL-6 and IL-8 when co-administered with TNF, although IL-17F was less potent than IL-17A. Dual neutralization of IL-17A and IL-17F demonstrated greater suppression of synoviocyte and healthy human dermal fibroblast activation and decreased expression of IL-6, IL-8, and metalloproteinase-3 (MMP3) than blockade of IL-17A or IL-17F alone. Of particular relevance to axSpA, IL-17F mediates osteogenic differentiation of human periosteal cells induced by supernatant from T_H17 or gamma-delta T cells and this can be inhibited using an antibody targeting IL-17F (19). Moreover, a bispecific antibody neutralizing both cytokines, bimekizumab, was more effective than antibodies targeting either IL-17A or IL-17F implying that such dual inhibition might prevent the ankylosis observed in patients with axSpA.

Relatively little is known about the pathophysiological role of other IL-17 family members relevant to axSpA. IL-17B may have an anti-inflammatory role by blocking IL-17E signaling during mucosal inflammation (20). IL-17C may have a similar role to IL-17A in anti-microbial protective responses in the intestine but is produced by epithelial rather than immune cells and may therefore provide a rapid local response to epithelial injury (21). A recent report indicated that IL-17D is the most highly expressed IL-17 family member in inflamed SpA synovium and is expressed by synovial stromal cells (22). The level of expression of IL-17D correlated with levels of expression of genes associated with synovial tissue remodeling (e.g. Bone Morphogenetic Protein (BMP) 4, BMP Receptor Type 1B, TGFβ Receptor 3, Wnt Family Members 3 and 11, Fibroblast

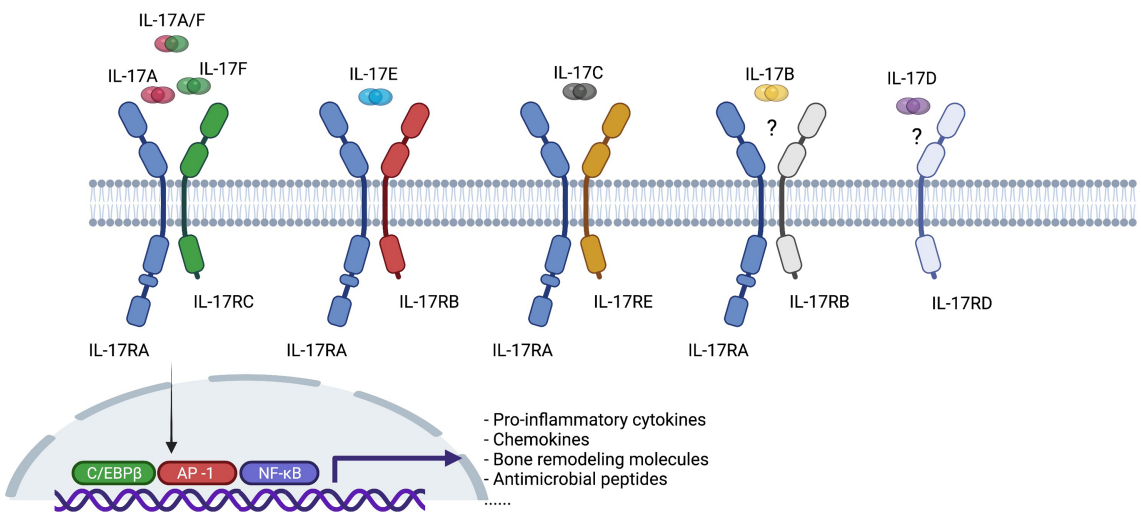

FIGURE 2 | Interleukin-17 (IL-17) cytokine family and interleukin-17 receptor (IL-17R) family. C/EBPβ, CCAAT-enhancer-binding protein beta; AP-1, activator protein-1; NK-κB, nuclear factor kappa B.

Growth Factor 1 and 17) and varied inversely with the degree of expression of genes associated with inflammation. IL-17D deficiency in mice increases joint and systemic inflammation suggesting that the cytokine has an anti-inflammatory role in joint inflammation. IL-17E is expressed by intestinal tuft cells and has a beneficial role in mucosal immunity to parasitic infection but has also been shown to inhibit autoimmunity induced by T_H17 cells and suppress production of IL-1, IL-6, and IL-23 by activated dendritic cells (23).

Case reports of patients with monogenic mutations in the IL-23/IL-17 pathway indicate that, physiologically, IL-23 and IL-17 are involved in maintaining mucosa barrier and protecting the body against bacterial and fungal infections. Increased risk for mucocutaneous candidiasis has been reported in patients with loss of function in IL-17F, IL-17RA or IL-17RC (24, 25). Patients with autoimmune polyglandular syndrome type 1 (APS-1) syndrome have neutralizing antibodies against IL-17A, IL-17F and/or IL-22, and have increased risk for candidiasis (26). Patients with autosomal dominant hyper-IgE syndrome (Job's syndrome, caused by *STAT3* mutation) have defects in IL-6, IL-23 and IL-22 signaling with reduced T_H17 cells, and are prone to have mucocutaneous candidiasis, staphylococcus aureus infection, and probably viral infections (27).

IL-17/IL-23 Pathway in Axial Spondyloarthritis

Human genome wide association analyses (GWAS) and studies in animal models and human tissues have implicated a pivotal role of IL-23/IL-17 pathway in the disease pathogenesis of ankylosing spondylitis (AS, a.k.a. radiographic axSpA, r-axSpA).

GWAS showed that *IL23R* (rs11209026, rs1004819, rs10489629, rs11465804, rs1343151, rs10889677, rs11209032, rs1495965) and *IL12B* (rs6556416, rs10045431) single nuclear polymorphism (SNPs) are associated with the susceptibility to AS, as well as SpA related conditions, such as psoriasis and inflammatory bowel disease (IBD) (28–30). In addition, GWAS of Vogt-Koyanagi-Harada (VKH) syndrome, a condition that primarily manifests as pan-uveitis, also showed that the *IL23R* SNP is associated with increased disease susceptibility (31). Apart from these two genes, genes that modulate the IL-23/IL-17 pathway, such as *STAT3* (rs6503695, rs744166), *IL1R2* (rs2310173), *CARD9* (rs10781500), have been reported to be associated with the risk of having AS and IBD (32). The SNP of *TNFAIP3*, the gene that encodes A20 protein and inhibits IL-17 signaling (33), was associated with risk for psoriasis (30).

Consistent with the genetic findings, *in vivo* over-expression of IL-23 in mouse models induces enthesitis, the pathologic hallmark of axSpA. In B10.RIII mice, IL-23 overexpression acts on a group of ROR-γt+ entheseal resident CD4 and CD8 negative T cells, and induces expression of IL-17 and IL-22, as well as IL-6 and Chemokine Ligand 1 (34). Additional features resembling the human axSpA phenotype included psoriasiform skin lesions, aortitis, uveitis, peripheral arthritis, and spondylitis. A subsequent report demonstrated that γδ T cells are the major cells producing IL17 in the enthesis of this IL-23 overexpressing model and that 50-80% of these cells are of the Vγ6+ phenotype (35). Furthermore, these cells also accumulate in the aortic valve and root as well as the ciliary body of the eye. However, this model has proven difficult to reproduce in other labs. In one report, over-expression of IL-23 using minicircle DNA led to chronic arthritis, severe bone loss, and myelopoiesis associated with the expansion of a myeloid lineage osteoclast precursor. This was partly dependent on TNF and IL-17A but could not be reproduced by overexpression of IL-17A (36). In SKG mice, after injection of curdlan, the mice developed IL-23 and T cell dependent arthritis and spondylitis, and the phenotype was transferrable by CD4+ T cells (37). Interestingly, a study in the HLA-B27 transgenic rat model with arthritis and spondylitis showed that IL-23R inhibition is effective for disease prevention when given prior to clinical onset, but when used for treatment of established disease, anti-IL-23R did not reduce clinical features or levels of IL-17 and IL-22 (38). Instead, when treated with anti-IL17A, the axial and peripheral joint inflammation were significantly reduced (39), suggesting IL-23 might be responsible for the onset of axSpA, but not for maintenance of the phenotype.

Immunohistochemical analysis of facet joints from patients with AS confirmed the presence of IL-23 expressing cells, including myeloperoxidase positive cells, macrophages and dendritic cells (40), as well as IL-17 positive neutrophils, T cells and mast cells (41). In peripheral blood of patients with AS, an increase of IL-23R expressing γδ T cells was also observed, associated with enhanced IL-17 secretion from these cells (42).

In summary, preclinical studies provided indirect evidence for inhibiting IL-23 or IL-17 as potential treatment options for axSpA.

CLINICAL STUDIES OF IL-17 INHIBITION IN AXIAL SPONDYLOARTHRITIS

Monoclonal antibodies against IL-17A alone or with dual inhibition of IL-17A/IL-17F have been developed and tested in clinical trials of axSpA, with clear efficacy in treating clinical symptoms and reducing inflammation.

Secukinumab

The first approved IL-17A antibody for AS was secukinumab (SEC), a fully human IgG1κ monoclonal antibody. MEASURE 1 and MEASURE 2 were two randomized, double-blind, placebo controlled, phase 3 trials in patients with active AS, defined by the modified New York criteria (see **Box 1**) (43), with prior exposure to no more than one tumor necrosis factor inhibitor (TNFi). Patients were randomized 1:1:1 into placebo, SEC 150mg every 4 weeks (Q4W), and SEC 75mg Q4W arms, with either intravenous loading dose in MEASURE 1 or subcutaneous loading dose in MEASURE 2. The primary endpoint was the Assessment of Spondyloarthritis International Society 20% response criterion [ASAS20 (44), see **Box 1**] at Week 16. Secondary endpoints included the ASAS40 response (44) (see **Box 1**), change of Bath AS disease activity index [BASDAI (45), see **Box 1**], SF-36, AS Quality of Life (ASQoL) scale, and ASAS partial remission. In MEASURE 1, 371 patients were randomized, with 61% of patients in the SEC 150mg arm, 60%

Box 1 | Fact Box. Diagnoses and major outcome measures in axial spondyloarthritis.

Ankylosing spondylitis (AS) – classified based on modified New York (mNY) criteria, composed of clinical criteria (inflammatory back pain (IBP), limited range of motion in lumbar spine, or limited range of motion in chest expansion) and radiological criterion (bilateral grade 2 sacroiliitis or unilateral grade 3 sacroiliitis on radiograph) (43). Definite AS is defined as meeting radiologic criterion and at least one clinical criterion.

Axial Spondyloarthritis (axSpA) – classified based on Assessment of SpondyloArthritis international Society (ASAS) criteria (44). In patients with 3 months back pain and age of onset < 45 years, axial spondyloarthritis is defined as 1) having sacroiliitis on imaging studies (either radiographic according to mNY criteria or magnetic resonance imaging (MRI) evidence of subchondral bone marrow edema highly suggestive of axSpA) and one spondyloarthritis (SpA) feature (IBP, arthritis, heel enthesitis, uveitis, dactylitis, psoriasis, Crohn's/colitis, good response to non-steroidal anti-inflammatory drugs, family history of SpA, positive HLA-B27, elevated C-reactive protein), or 2) positive HLA-B27 and two other SpA features. The condition is further categorized as radiographic axSpA (r-axSpA) if sacroiliitis is present on pelvis radiograph, and non-radiographic axSpA (nr-axSpA) if not present.

ASAS 40% response criteria (ASAS40) – improvement of ≥ 40% and ≥2 units (0-10 scale) in at least three of the four domains (patient global, pain, function, inflammation), and no worsening in any domain (44).

Bath ankylosing spondylitis disease activity index (BASDAI) – a six-question (each with 0-10 scale), self-administered questionnaire, assessing fatigue, spinal pain, peripheral arthritis, enthesitis, intensity and duration of morning stiffness in patients with AS (45).

Bath ankylosing spondylitis functional index (BASFI) - a 10-question (each with 0-10 scale), self-administered questionnaire, assessing degree of functional limitations in patients with AS (46).

Ankylosing spondylitis disease activity score (ASDAS) - a composite score, including assessment of total back pain, patient global of disease activity, peripheral pain and swelling, duration of morning stiffness, and C-reactive protein or erythrocyte sedimentation rate (47).

Spondyloarthritis Research Consortium of Canada MRI index for scoring inflammation in the sacroiliac joints (SIJ) (SPARCC MRI SIJ score) - a semi-quantitative scoring system to assess active inflammation in SIJs, based on the presence, intensity and depth of bone marrow edema on a fat-suppressed sequence in six consecutive coronal slices of pelvis MRI, with a total maximum score of 72 (48).

Spondyloarthritis Research Consortium of Canada MRI index for scoring inflammation in the spine (SPARCC MRI Spine score) - a semi-quantitative scoring system to assess active inflammation in the spine, based on the presence, intensity and depth of bone marrow edema on a fat-suppressed sequence of 3 consecutive sagittal slices through each discovertebral level of the entire spine, with a total maximum score of 414 (49). Modified Stoke AS Spine Score (mSASSS) - a semi-quantitative scoring system to assess structural damage of the spine based on certain features (erosion, sclerosis, squaring, syndesmophytes and bony bridging between adjacent vertebra) of the anterior vertebral corners on lateral projections of cervical and lumbar spine radiographs, with a total maximum score of 0 -72 (50).

of patients in the SEC 75mg arm *versus* 29% of patients in the placebo arm meeting the primary endpoint. ASAS40 responses were 42%, 33% and 13% in these three arms, respectively. In MEASURE 2, 219 patients were randomized, and 61% of patients in the SEC 150mg arm, 41% of patients in the SEC 75mg arm, *versus* 28% of patients in the placebo arm met the primary endpoint. ASAS40 responses were 36%, 26% and 11% in these three arms, respectively (51) (**Table 1**). All other secondary endpoints were met, except ASAS partial remission in the MEASURE 2 study. Long term extension studies of SEC up to 5 years follow up showed that, for those who stayed on SEC for two years, 84% patients (N = 230/274) remained on SEC at Year 5, with an ASAS40 response rate of 65.2% and BASDAI of 2.6, indicating a very low level of active symptomatology (52).

A 1-year placebo-controlled RCT of SEC recruited 555 patients with non-radiographic axial SpA (nr-axSpA) who were randomized (1:1:1) to receive subcutaneous SEC 150 mg with a loading dose (loading dose [LD] group), SEC 150 mg without a loading dose (non–loading dose [NL] group), or placebo weekly and then Q4W starting at week 4 (53). The primary endpoint was the ASAS40 response at week 16 (European Union and non-US analysis) and week 52 (US analysis). Escape to open label SEC was possible at any time after week 20. Most recruited patients were naïve to TNFi (90.3%) and in these patients an ASAS40 response was achieved at 16 weeks in 41.5% of the SEC 150mg LD group, 42.2% of the SEC NL group, and 29.2% of those on placebo (**Table 1**). The corresponding response rates at week 52 were 35.4%, 39.8%, and 19.9% of patients in the SEC 150mg LD, SEC 150mg NL, and placebo groups, respectively. All major secondary endpoints showed greater improvement in the SEC groups compared to those on placebo at week 16. These included the ASAS5/6 response, ASAS partial remission, BASDAI50 response at weeks 16, change from baseline to week 16 in the BASDAI, high sensitivity C-reactive protein (CRP), Bath AS Functional Index [BASFI (46), see **Box 1**], MRI SIJ edema (Berlin score), SF-36 Physical Component Scale, and ASQoL score. For the week 52 analysis of secondary endpoints, significance *versus* placebo was achieved for the BASDAI50 and AS Disease Activity Score [ASDAS (47), see **Box 1**] inactive disease [(SDAS-ID, defined as an ASDAS score <1.3) responses in the SEC 150mg NL group but not for ASDAS-ID in the SEC LD group and in view of the hierarchical testing procedure SIJ edema score on MRI and ASQoL for the 150 mg LD group were not tested. The frequencies of serious adverse events (SAE) and discontinuations due to adverse events were similar across the three groups. There were 14 cases of uveitis in 11 patients, 9 in the SEC groups (4 *de novo* cases) and 2 in the placebo group. Seven patients receiving SEC reported colitis (5 Crohn's disease and 2 ulcerative colitis) of whom two had a history of colitis.

Overall, the SEC RCT data demonstrates convincingly that IL-17A targeted therapy with this agent is effective at ameliorating inflammation in AS as well as nr-axSpA patients who are biologic disease modifying anti-rheumatic drug (bDMARD) naïve and refractory to non-steroidal anti-inflammatory drug (NSAIDs) therapy, but the trials recruited insufficient numbers of TNFi experienced patients. Moreover, a trial recruiting only TNFi experienced patients has not yet been reported. The impact on concomitant musculoskeletal inflammation in the peripheral skeleton in axSpA, such as enthesitis and peripheral synovitis, has also not yet been reported.

Ixekizumab

Ixekizumab (IXE) is another IL-17A inhibitor that have been approved in treating active axSpA. It is a humanized IgG4 monoclonal antibody. In the COAST-V study, a phase 3, double-blinded RCT, biologic naïve patients with active r-axSpA based on ASAS classification criteria (44) (see **Box 1**) were included in the study (54). In addition to the placebo control, the study also had an active comparator arm of adalimumab 40mg Q4W, a fully human

TABLE 1 | Pivotal randomized clinical trials of IL-17 inhibitors in axial spondyloarthritis.

Study ID	Drug	Acronym	Clinical Trial Phase	Condition	Previous bDMARD use	Primary Endpoint	TNF inaïve (%)	Arms	Enrollment (n)	Meeting Primary Endpoint
NCT01358175	SEC	MEASURE 1	Ph. 3	AS	Not more than one TNFi	W16 ASAS20	73%	Placebo	122	29%
							73%	SEC IV loading + 150mg Q4W	125	61%
							74%	SEC IV loading + 75mg Q4W	124	60%
NCT01649375	SEC	MEASURE 2	Ph. 3	AS	Not more than one TNFi	W16 ASAS20	61%	Placebo	74	28%
							61%	SEC IV loading + 150mg Q4W	72	61%
							62%	SEC IV loading + 75mg Q4W	73	41%
NCT02696031	SEC	PREVENT	Ph. 3	nr-axSpA	Prior TNFi allowed	W16 ASAS40*	91.9%	Placebo	186	29.2%
							88.6%	SEC LD + 150mg Q4W	185	41.5%
							90.2%	SEC NL + 150mg Q4L	184	42.2%
NCT02696798	SEC	COAST-V	Ph. 3	AS	Not allowed	W16 ASAS40	100%	Placebo	87	18%
							100%	ADA 40mg Q2W	90	32%
							100%	IXE 80mg Q2W	83	52%
							100%	IXE 80mg Q4W	81	48%
NCT02757352	IXE	COAST-W	Ph. 3	r-axSpA	TNFi experienced required	W16 ASAS40	0%	Placebo	104	12.5%
							0%	IXE 80mg Q2W	98	30.6%
							0%	IXE 80mg Q4W	114	25.4%
NCT02696785	IXE	COAST-X	Ph. 3	nr-axSpA	Not allowed	W16 ASAS40*	100%	Placebo	105	19%
							100%	IXE 80mg Q2W	102	41%
							100%	IXE 80mg Q4W	96	35%
NCT02963506	BKZ	BE AGILE	Ph. 2B	AS	TNFi experienced allowed	W12 ASAS40	88.3%	Placebo	60	13.3%
							86.9%	BKZ 16mg Q4W	61	29.5%
							88.5%	BKZ 64mg Q4W	60	42.6%
							88.3%	BKZ 160mg Q4W	60	46.7%
							91.8%	BKZ 320mg Q4W	61	45.9%
NCT02763111	NTK	–	Ph. 2	AS	TNFi experienced allowed	W16 ASAS20	81.8%	Placebo	22	42.9%
							81.8%	NTK 40mg	22	72.7%
							90.9%	NTK 80mg	22	81.8%
							86.4%	NTK 120mg	22	90.9%

SEC, secukinumab; IXE, ixekizumab; BKZ, bimekizumab; NTK, netakimab; AS, ankylosing Spondylitis; nr-axSpA, nonradiographic axial spondyloarthritis; r-axSpA, radiographic axial spondyloarthritis; bDMARD, biologic disease modifying anti-rheumatic drug; TNFi, tumor necrosis factor inhibitor; ASAS20, the Assessment in Ankylosing Spondylitis 20% response criteria; ASAS40, the Assessment in Ankylosing Spondylitis 40% response criteria.

IgG1 monoclonal antibody to TNFα. The treatment arms included IXE 80mg every 2 weeks (Q2W) and IXE 80mg Q4W. The primary endpoint of the study was the proportion of patients who achieved an ASAS40 response at Week 16. The main secondary endpoints included the ASAS20 response, the BASDAI50 response, change in ASDAS, change in BASFI, and ASDAS-ID. In this study, MRI of spine and SIJ were obtained at baseline and Week 16, and change in the severity of MRI inflammation in the spine was assessed using the Spondyloarthritis Research Consortium of Canada (SPARCC) MRI spine score (see **Box 1**)while change in the severity of MRI inflammation in the sacroiliac joints was assessed using the SPARCC MRI SIJ score (see **Box 1**) and these were among the secondary outcomes (48, 49). A total of 341 patients were included and randomized 1:1:1:1 into 4 arms. At Week 16, significantly more patients in the IXE Q2W arm (51.8%), the IXE Q4W arm (48.1%), and the adalimumab arm (35.6%) achieved an ASAS40 response, compared to patients in the placebo arm (18.4%) (**Table 1**). The main secondary outcomes were significantly better in the active treatment compared to placebo arms, including the SPARCC MRI spine and SIJ scores. During the 16 weeks of the double blinded period, the most common AEs were nasopharyngitis, which occurred in 23 patients, and almost equally among the 4 arms (6.0% to 7.4%). One patient in each IXE arm, and 3 in the adalimumab arm experienced SAE. Three patients in the IXE Q2W arm and one in the adalimumab arm discontinued the study due to AEs. One case of candida infection in the adalimumab arm was reported, as well as one case of cerebrocardiovascular event in the IXE Q4W arm, one case of IBD in the IXE Q2W arm, and one case of depression in the adalimumab arm.

The COAST-W study of IXE followed a similar design to COAST-V, except that patients in COAST-W were required to have discontinued at least one TNFi, but not more than 2 TNFi, due to inadequate response or intolerance (55). A total of 316 patients were included and randomized 1:1:1 into placebo, IXE 80mg Q2W and IXE 80mg Q4W arms. At Week 16, significantly more patients in the IXE Q2W arm (30.6%) and the IXE Q4W arm (25.4%) achieved ASAS40, compared to placebo (12.5%) (**Table 1**). A proportion of patients had spine MRI at baseline and Week 16. The reductions in the MRI SPARCC spine scores were significantly more in the IXE Q2W and the IXE Q4W arms than in the placebo arm (**Table 2**).

Sustained efficacy was observed at Week 52 for both COAST-V and COAST-W trials (56). At Week 52, 47% - 53% of patients in the COAST-V trial and 31% - 39% patients in the COAST-W trial achieved an ASAS40 response and demonstrated sustained clinical efficacy of IXE. Moreover, patients originally randomized to placebo showed rapid improvement in ASAS40 response rates after switching to IXE and by 52 weeks responses were similar to those seen in patients originally randomized to IXE. In the COAST-V trial, patients originally randomized to Adalimumab demonstrated further improvement after switching to IXE with ASAS40 response of 36% at week 16 increasing to 51.2% at week 52. Treatment with IXE was well tolerated in this

TABLE 2 | Spine or pelvis MRI score changes in IL-17i trials in axial spondyloarthritis.

Study ID	Drug	Acronym	MRI endpoint	Arms	Number of patients with MRI	Baseline	Changes
NCT02696031	SEC	PREVENT	Berlin SIJ MRI score change	Placebo	139	2.70 (3.96)	-0.59
				SEC LD + 150mg Q4W	132	2.80 (3.83)	-2.38
				SEC NL + 150mg Q4L	134	2.24 (3.29)	-1.42
NCT02696798	IXE	COAST-V	SPARCC Spine Score change from baseline to W16	Placebo	87	15.8 (21.2)	-1.51 (1.15)
				ADA 40mg Q2W	90	20.0 (28.4)	-11.57 (1.11)
				IXE 80mg Q2W	83	16.6 (23.8)	-9.58 (1.17)
				IXE 80mg Q4W	81	14.5 (20.6)	-11.02 (1.16)
			SPARCC SIJ Score change from baseline to W16	Placebo	87	5.0 (9.6)	0.9 (0.6)
				ADA 40mg Q2W	90	4.7 (11.2)	-4.2 (0.6)
				IXE 80mg Q2W	83	6.4 (10.9)	-4.3 (0.6)
				IXE 80mg Q4W	81	4.5 (9.1)	-4.0 (0.6)
NCT02757352	IXE	COAST-W	SPARCC spine Score change from baseline to W16	Placebo	51	6.4 (10.2)	3.3 (1.4)
				IXE 80mg Q2W	58	11.1 (20.3)	-4.0 (1.5)
				IXE 80mg Q4W	53	8.3 (16)	-3.0 (1.4)
NCT02696785	IXE	COAST-X	SPARCC SIJ Score change from baseline to W16	Placebo	105	6.2 (9.1)	-0.31 (0.54)
				IXE 80mg Q2W	102	7.5 (10.8)	-4.52 (0.53)
				IXE 80mg Q4W	96	5.3 (8.3)	-3.38 (0.55)
NCT02963506	BKZ	BE AGILE	SPARCC SIJ Score change from baseline to W12	Placebo	20	NR	-3.7
				BKZ 16mg Q4W	20	NR	-10
				BKZ 64mg Q4W	20	NR	-9.5
				BKZ 160mg Q4W	20	NR	-2.5
				BKZ 320mg Q4W	20	NR	-5.5
			Berlin Spine MRI score change from baseline to W12	Placebo	20	NR	-1.3
				BKZ 16mg Q4W	20	NR	0.5
				BKZ 64mg Q4W	20	NR	-1.8
				BKZ 160mg Q4W	20	NR	-3.5
				BKZ 320mg Q4W	20	NR	-3.1

SEC, secukinumab; IXE, ixekizumab; BKZ, bimekizumab; SPARCC, Spondyloarthritis Research Consortium of Canada; SIJ, sacroiliac joint; NR, not reported.

52-week extension. Serious infections were reported by 3 patients in COAST-V and 3 patients in COAST-W but only 1 patient withdrew from the study. Candida infection was reported by 2 patients in each of the COAST-V and COAST-W studies. Two patients reported *de novo* Crohn's in COAST-V and 2 reported flares of ulcerative colitis but there was no association between these events and length of exposure to IXE. There were no events related to colitis in COAST-W. Acute anterior uveitis (AAU) was reported by 6 patients (1.8%) in COAST-V and 11 (3.9%) in COAST-W of whom 14 had a history of AAU. Corresponding exposure adjusted incidence rates (IRs, number of events per 100 patient years) for Crohn's disease and ulcerative colitis were 0.8 and 0.4, respectively, while the exposure adjusted IR for AAU was 3.9. These event rates are comparable to those reported previously for these extra-articular manifestations in patients receiving TNFi bDMARDs for AS (57, 58).

IXE has also demonstrated efficacy in a 52-week placebo-controlled trial of nr-axSpA (COAST-X) (59). Patients refractory to ≥2 NSAIDs with either elevated CRP or MRI inflammation in the SIJ and meeting ASAS classification criteria for axSpA were randomized 1:1:1 to IXE 80mg Q4W, Q2W or placebo. Escape to open label IXE Q2W was possible after week 16 at investigator discretion. Primary endpoint was the ASAS40 response at weeks 16 (European Union and non-US analysis) and week 52 (US analysis). It was achieved at 16 weeks by 35%, 40%, and 19% of patients in the IXE 80mg Q4W, IXE 80mg Q2W, and placebo groups, respectively (**Table 1**). At 52 weeks, the corresponding ASAS40 response rates were lower at 30%, 31%, and 13%. However, 25% on ixekizumab Q4W, 17% on ixekizumab Q2W, and 6% on placebo had ASAS40 at their last visit before switching. Statistically significant group differences were seen as soon as week 1 and all major secondary endpoints showed greater improvement in the IXE groups compared to those on placebo. These included the ASDAS, BASDAI, SF-36 Physical Component Scale, and SPARCC MRI SIJ inflammation score (**Table 2**). The frequencies of SAE, discontinuations due to adverse events, and frequency of anterior uveitis were similar across the three groups. A single case of Crohn's disease occurred in a patient reported as having pre-existing diarrhea.

In a withdrawal study (COAST -Y), patients in the previous COAST clinical trial program who completed 52 weeks of treatment with IXE continued a further 24 weeks on IXE, and those who achieved ASDAS-ID were randomly assigned to continue IXE Q2W, Q4W or to receive placebo (60). The primary endpoint was the proportion of patients who had not flared, which was defined as an ASDAS≥2.1 at two consecutive visits or an ASDAS>3.5 at any visit, at the 40-week time point of the randomized withdrawal period, with time-to-flare as a major secondary endpoint. A total of 773 patients were enrolled and 155 were randomized to treatment withdrawal or continuation on treatment with IXE. At 40 weeks, 83.3% of those who continued treatment with IXE had not flared as compared to 54.7% of those on placebo, and IXE significantly delayed time to flare. Re-treatment of patients who flared with at least 16 weeks of open-label IXE resulted in recapture of ASDAS-ID in only 44% of those who had withdrawn to placebo.

Overall, the IXE RCT data demonstrated convincingly that IL-17A targeted therapy with this agent is effective at ameliorating inflammation in r-axSpA as well as nr-axSpA and in bDMARD naïve as well as TNFi experienced patients. It has a clearly beneficial impact on objective manifestations of disease, namely, the CRP and MRI inflammation in the SIJ and spine, but impact on concomitant musculoskeletal inflammation in the peripheral skeleton, such as enthesitis and peripheral synovitis, has not yet been reported in axSpA. An important consideration is that disease flare occurs upon treatment withdrawal in about half of patients over a 40-week time frame and re-institution of therapy recaptures the response in only half of the patients that flare.

Bimekizumab

Bimekizumab (BKZ) is a monoclonal humanized IgG1 antibody that neutralizes both IL-17A and IL-17F. Its efficacy in active AS was investigated in a phase 2b placebo controlled, double blinded, dose ranging trial (61). The primary endpoint was the proportion of patients who achieved the ASAS40 at Week 12. Secondary endpoints included the ASAS20 response, change in the BASDAI, change in the BASFI, and change in the ASDAS at Week 12. A total of 303 patients were randomized 1:1:1:1:1 into placebo, BKZ 16mg, 64mg, 160mg, or 320mg Q4W. At Week 12, more BKZ-treated patients achieved an ASAS40 response compared to placebo (29.5% - 46.7% *vs* 13.3%, $p < 0.05$ in all comparison, **Table 1**). In addition, a significant dose-response was observed. In patients treated with BKZ, the ASAS20 response at Week 12 was achieved by 41% (BKZ 16mg Q4W) to 72.1% (BKZ 320mg Q4W) of patients, compared to 28.3% in the placebo arm. Efficacy was maintained at 48 weeks, and 58.6% patients in the BKZ 160mg Q4W arm and 62.3% patients in the BKZ 320mg Q4W arm achieved the ASAS40 response. During the 48-week study period, the most reported adverse event was nasopharyngitis, with 34 cases out of 303 patients. Candidiasis infection was reported in 19 patients, major cardiovascular events in 2 patients, and IBD in 4 patients, two being *de novo* events. No malignancy or suicidal ideation was reported. Thirteen patients had SAE, and 20 patients discontinued the study drug due to AEs. Some patients in the study had MRI spine at baseline and at Week 12. In addition to the symptom relief, inflammatory lesions seen on spinal and SIJ MRI were reduced after 12 weeks of BKZ (**Table 2**).

Overall, one can conclude that treatment with BKZ is efficacious and well-tolerated though it is questionable whether clinical and MRI improvement in disease activity is superior to responses observed with bDMARDs targeting only IL-17A. While different patient populations recruited to these clinical trials requires caution in making comparisons between outcomes in different clinical trials, it nevertheless appears unlikely that the dual inhibition hypothesis is supported by the results of the efficacy data related to clinical outcomes in this phase 2b trial of BKZ. An important future priority will be to determine whether this agent might prevent the development of ankylosis, which would support the dual inhibition hypothesis and be consistent with the *in-vitro* data that blockade of both IL-17A and IL-17F is more effective at preventing osteogenic differentiation of periosteal stem cells than blockade of either cytokine alone.

Netakumab

Netakumab (NTK) is a recombinant humanized IgG1 IL-17A monoclonal antibody with modified CDR-regions and Fc-fragment. In a phase 2, double blinded RCT, patients with active AS were randomized 1:1:1:1 to placebo *vs.* NTK 40mg, 80mg, 120mg Q2W (62). The primary endpoint was the percentage of patients achieving the ASAS20 response at Week 16. The ASAS40 response at Week 16 was assessed as one of the secondary endpoints. A total of 88 patients were included in the study, with 22 patients in each arm. At Week 16, 72.7%, 81.8% and 90.9% patients in the NTK 40mg, 80mg, and 120mg arms achieved an ASAS20 response, compared to 42.8% patients in the placebo arm, and significantly more in the NTK 80mg and 120mg arms (**Table 1**). An ASAS40 response was achieved in 40.9%, 63.6% and 72.7% patients in the NTK 40mg, 80mg, and 120mg arms respectively, compared to 14.3% in the placebo arm. No SAE or withdrawal due to AE was observed during the 16-week study period. However, it was a relatively small study with a short study duration. More data is needed to demonstrate its efficacy and safety in treating patients with axSpA.

Safety of IL-17 Inhibition

Although patients with monogenic diseases with loss of IL-17 have increased risk for candidiasis, monoclonal antibodies against IL-17A seem to be relatively safe. Using post-marketing surveillance data and pooled clinical trial data, exposure adjusted IR of adverse events of SEC in patients with AS per 100 patient-years was 1.2 for serious infections, 0.7 for candida infections, 0.6 for major adverse cardiac events, 0.4 for Crohn's disease, 0.2 for ulcerative colitis, and 0.5 for malignancy (63). IXE reported a similar safety profile from pooled clinical trial data and showed that the adjusted IR per 100 patient-years was 1.3 for serious infections and infestations, 1.6 for candida infection, 0.1 for major adverse cardiac events, 0.5 for Crohn's disease, 0.4 for ulcerative colitis, 0.4 for malignancy (64). No reactivation of tuberculosis was observed in these two studies, and demyelinating disease was not commented.

Real-World Data

Consistent with the efficacy and safety data from clinical trials, in clinical practice, IL-17 inhibitor was shown to be effective. In patients with axSpA started on SEC as part of routine clinical care (N =1860), the drug retention rate at 12 months was reported to be 72%. Twenty two percent of patients reached ASAS40 and 26% had ASDAS major improvement (ASDAS-MI, ASDAS decrease ≥2.0). When drug retention was examined in TNFi naïve AS patients, the drug retention rate of SEC at 12 months was increased to 84%, and 55% of patients reached ASAS40 and 51% had ASDAS-MI at 12 months (65).

CLINICAL STUDIES OF IL-17 IN SPONDYLOARTHRITIS RELATED DISEASES

Genetic and epidemiologic studies have established the association between AS and psoriasis, uveitis, and IBD. More than one third of patients with AS report one or more extra-articular manifestations, including psoriasis, uveitis and IBD (66). However, despite the shared genetic risk factors in the IL-23/IL-17 pathway, and a positive response to IL-23 inhibitors in psoriasis/PsA and IBD, the efficacy of IL-17i in SpA-related conditions varies.

Phase 3 clinical trials of SEC, IXE and BKZ have demonstrated clear efficacy of IL-17i in psoriasis and PsA. Among them, the MAXIMISE trial is a phase 3b, randomized, double blind trial that focuses on axial PsA, a condition that may be distinct from axSpA, but with overlapping features (67). In this study, patients with PsA fulfilling Classification criteria for Psoriatic Arthritis (CASPAR) criteria, with active spinal symptoms [defined as BASDAI ≥ 4 and spinal pain ≥ 40mm (0-100mm visual analog scale)], and inadequate response to NSAIDs were included. A total of 498 patients were randomized 1:1:1 into SEC 300mg Q4W *vs* SEC 150mg Q4W *vs.* placebo arms. Two-third of the patient population had Grade 1 to Grade 4 sacroiliitis on either side as determined by the local investigator, although the number meeting mNY criteria for radiographic sacroiliitis was not stipulated. Around 60% of patients had a positive MRI with inflammation in the spine and SIJs, although the criteria used to define this were not stipulated. Moreover, Berlin scores for spinal inflammation at baseline were less than half of those recorded in a trial of a TNFi agent (certolizumab) in patients with r-axSpA and even less than in those patients with nr-axSpA. HLA-B27 status was positive for 33% of the 261 patients for whom this data was available (68). The primary endpoint was the proportion of patients achieving ASAS20 response at Week 12, and secondary endpoints included the ASAS 40 response at Week 12. At Week 12, 63% patients in the SEC 300mg Q4W arm and 66% patients in the SEC 150mg Q4W arm achieved an ASAS20 response, compared to 31% in the placebo arm. ASAS40 response rates were 44%, 40% and 12% respectively. At Week 52, the retention rates were 83% for SEC 300mg Q4W arm and 86% for SEC 150mg Q4W arm. The study also included exploratory outcomes such as improvement of Berlin MRI score for spine and SIJ at Week 12. Although the baseline MRI scores for spine and SIJ were relatively low, the least square means of difference from baseline to Week 12 between SEC arms and the placebo arm were significant. However, the magnitude of response was substantially less than observed in an RCT of a TNFi in patients with r-axSpA and nr-axSpA (68). A significant drawback of this study is the lack of central evaluation of radiographs and lack of detail as to how MRI positivity for inflammation in the spine was defined. The age of the patients was higher than typically noted for AS trials so it is likely that many patients will have had MRI inflammation related to degenerative changes in the spine and SIJs. The 33% prevalence of HLA-B27 in this population was lower than the 60% previously reported in psoriatic axSpA further suggesting that some patients may have had mechanical causes of back pain and MRI inflammation. Moreover, 80% had concomitant peripheral arthritis and the self-reported improvement in clinical outcomes of pain and stiffness could reflect predominant alleviation of peripheral arthritis. However, the improvement in MRI inflammation in

SEC-treated patients supports the hypothesis that IL-17A is a cytokine that also mediates inflammation in the axial joints of patients with PsA.

In contrast, the efficacy of IL-17 blockade in uveitis has been inconclusive and the most recent data indicates it is not effective. Subcutaneous loading and maintenance dosing of SEC were tested in three phase 3 randomized, double-blind trials for Behcet's uveitis, active non-Behcet's, noninfectious uveitis, and quiescent, non-infectious, non-Behcet's uveitis, respectively, and none of the studies met its primary endpoint for efficacy (69). However, in a proof-of-concept phase 2 study with 37 patients, when given intravenously at 10mg/kg for loading and maintenance, SEC seemed effective compared to subcutaneous dosing and placebo (70). It is worth noting that the sample size of this study was very small, and all the uveitis trials only included patients with intermediate, posterior, and pan-uveitis, whereas in patients with AS, anterior uveitis is much more common. On the other hand, from the safety perspective, IL-17 blockade does not appear to trigger uveitis flares in patients with AS. In a pooled analysis of three phase 3 RCTs of SEC in AS, the exposure adjusted IR for uveitis was 1.4 per 100 patient-years. In comparison, the exposure adjusted IR of uveitis during TNFi treatment was 2.6 to 3.5 per 100 patient-years (71). Caution is warranted in comparing event rates across trials because event rate during a trial will depend on the numbers of patients recruited with a prior history of uveitis, especially in the year preceding entry into the trial. A recent study reporting AAU event rates in the Swedish Rheumatology Quality Register was based on 4851 treatment starts (456 secukinumab; 4395 any TNFi); the rate of AU-diagnoses per 100 patient-years was 6.8 (95% CI 5.2 to 8.7) for secukinumab (72). Among the TNFi, the rate varied from 2.9 (95% CI 2.1 to 3.7) for infliximab and 4.0 (95% CI 3.3 to 4.9) for adalimumab to 7.5 (95% CI 6.7 to 8.4) for etanercept. Sensitivity analyses confirmed the pattern of higher AU rates with secukinumab and etanercept *versus* monoclonal TNFi.

For Crohn's disease, it is surprising that IL-17A blockade is not only ineffective, but may potentially worsen disease activity and/or cause serious side effects. A phase 2 study of SEC in patients with active Crohn's disease was terminated prematurely because it met the prespecified futility criteria (73). Another phase 2 study, which evaluated AMG 827 (Brodalumab), an anti-IL-17 receptor antibody, did not demonstrate efficacy either (74). Instead, in both studies, a disproportionate number of patients in the IL-17 blockade arms experienced worsening of their Crohn's disease compared to placebo. Furthermore, in the SEC study, more patients experienced adverse events in the SEC arm (74%, 29/39) than in the placebo arm (50%, 10/20), particularly infections.

It is possible that the greater disease severity, as defined by higher rates of prior bowel surgery and failure of TNFi therapy, in patients randomized to SEC were contributory factors. More likely, these results suggest a different role of IL-17A in IBD, specifically, protective rather than pathogenic. In mice, IL-17A or IL-17 receptor deficient T cells induce flare of colitis when transferred into RAG-1 deficient mice, and blocking IL-17A increases tissue damage and leads to enhanced inflammation (75–77). Furthermore, it has been shown that IL-17A is produced in an IL-23 independent fashion by resident γδ T cells in the intestinal lamina propria and these cells have an important role in maintaining the mucosal barrier (77). These findings in murine models probably explain the unsuccessful clinical trials of IL-17A inhibitors beyond trial design. Interestingly, IL-17F deficient mice, but not IL-17A deficient mice, were found to be resistant to chemically induced colitis and T cell induced colitis, with increased T_{reg} population in colon, commensal dysbiosis (increased *Clostridium* cluster XIVa, reduced *Prevotellaceae*) and reduced expression of some antimicrobial peptides (78). Antibody against IL-17F was effective in treating chemically induced colitis, while antibody to IL-17A was not. Whether sole IL-17F inhibition would be effective in IBD patients is unclear.

What is reassuring is that, clinically, in patients with psoriasis, PsA or AS treated with SEC or IXE, the incidence of IBD was low, and the risk was similar to patients without this exposure (79–81). A recent retrospective pooled analysis of 7355 patients and a cumulative exposure time of 16,226.9 patient-years described the incidence rates of IBD in patients enrolled in 21 clinical trials of SEC for the treatment of psoriasis, PsA, and AS (81). There were 41 cases of IBD. Forest plots illustrating the relative risk of Crohn's and UC *versus* placebo did not show any increased risk from treatment with SEC. There was also no evidence of a dose-response relationship between SEC dose (150 mg *vs* 300 mg) and rates of reported IBD and the exposure adjusted IRs did not increase over time for each patient or each indication. In the post-marketing safety surveillance analysis, the cumulative reporting rate of IBD remained stable at approximately 0.20 reported events per 100 PY over a cumulative exposure of >96,000 patient years.

Further, a recent study examined the intestinal biopsy samples from axSpA patients who developed clinical Crohn's disease after IL-17A inhibition, and found an expansion of IL-17E producing cells (82). Whether a similar increase, or even higher levels might be observed in those without colonic inflammation is unknown. In contrast, in classic Crohn's disease patients, the expression of IL-17E were reduced, and the reduction correlated with endoscopic severity, suggesting IL-17E, similar to IL-17A, might be protective in patients with Crohn's disease (83).

These differences, both at the clinical level, and at the translational level, suggest that the underlying mechanism of IL-17A inhibition induced colonic inflammation might be different from Crohn's disease.

CLINICAL STUDIES OF IL-23 INHIBITION IN AXIAL SPONDYLOARTHRITIS

Risankizumab

A proof-of-concept dose-ranging placebo-controlled RCT evaluated risankizumab (BI 655066/ABBV-066), a humanized, IgG1 monoclonal antibody that selectively targets the p19 subunit of IL-23, in patients with definite AS refractory to ≥ 2 NSAIDs (84). Radiographic sacroiliitis was determined by local

reader evaluation. The dosing range of risankizumab had previously demonstrated efficacy in RCTs of psoriasis, PsA, and Crohn's disease (85–88). A total of 159 patients were randomized 1:1:1:1 to risankizumab (18 mg single dose, 90 mg or 180 mg at day 1 and weeks 8, 16 and 24) or placebo for 24 weeks with escape treatment to 180 mg risankizumab being available at 16 weeks for patients not achieving an ASAS20 response at 12 weeks. Primary endpoint was the ASAS40 response at 12 weeks and this was achieved by 25%, 21% and 15% of patients in the 18 mg, 90 mg and 180 mg risankizumab groups, respectively, which was not significantly different from the placebo group (18%). A total of 96 (60.4%) patients switched to the escape 180mg risankizumab treatment arm and prolongation of treatment for up to 40 weeks did not substantially improve ASAS40 response rates. No significant differences were noted for ASAS20, ASAS5/6, or ASAS partial remission responses. There was a dose-dependent reduction in the CRP level and greater reduction in the ASDAS-CRP for the 18mg and 180mg Risankizumab groups compared to placebo. However, a clinically relevant reduction in ASDAS-CRP ≥ 1.1 was not achieved with risankizumab and the reduction was primarily driven by the change in CRP. Greater though modest reduction in SPARCC MRI spine inflammation scores for the risankizumab 90mg and 180mg groups when compared to placebo was noted at week 12 in patients with a clinical response. But SPARCC MRI scores at 12 weeks were no different among groups who switched to escape treatment. There were no differences between treatment groups in levels of the IL-23/T_H17 pathway biomarker β-defensin 2 or in several biomarkers of bone remodeling (dikkopf-1, sclerostin, BMP-7 and osteocalcin). Treatment was well tolerated with no significant differences in adverse events between treatment groups and no reports of severe infections.

Ustekinumab

Three RCTs have evaluated ustekinumab, a human monoclonal antibody targeting the p40 subunit found in both IL-12 and IL-23, after an open label pilot study of 20 patients with AS (TOPAS) demonstrated improvement in clinical and MRI parameters of spinal inflammation at 24 weeks after receiving ustekinumab 90mg at 0, 4, and 16 weeks (89). This agent is approved for the treatment of psoriasis, PsA, and Crohn's disease, and improvement in spinal symptoms was reported in a subgroup of patients with PsA considered to have axial inflammation by their physician. All three RCTs evaluated ustekinumab at 45mg or 90mg *versus* placebo at weeks 0, 4, 16 and randomized 1:1:1 to these groups (90). Two studies recruited patients with active r-axSpA. One of these studies included patients refractory to ≥2 NSAIDs but naïve to bDMARD therapy while the second study included patients refractory to a single TNFi. A third study recruited patients with nr-axSpA refractory to ≥2 NSAIDs and could have been exposed to no more than one TNFi. The primary endpoint was the ASAS40 response at week 24 for the r-axSpA trials and the ASAS20 response at week 24 for the nr-axSpA trial. Patients failing to achieve ≥10% improvement from baseline in both total back pain and morning stiffness measures at both week 12 and week 16 could escape at week 16 to either open label golimumab (patients with r-axSpA) or were re-randomized in a blinded manner to ustekinumab 45mg or 90mg (patients with nr-axSpA). The presence of radiographic sacroiliitis and MRI inflammation in the SIJ typical of axSpA was assessed by a single central reader. Major secondary endpoints included the BASDAI50 response, ASDAS-CRP inactive disease, and change from baseline in the BASFI. Study continuation for all 3 studies was based on the week 24 results in 346 patients with r-axSpA who were naïve to bDMARD. This showed that primary and secondary endpoints were not met and so all 3 studies were discontinued. In particular, there was no significant difference in the proportions of patients who achieved an ASAS40 response in the ustekinumab 45 mg (31%) and 90 mg (28%) groups when compared to the placebo group (28%). There were also no significant differences in MRI inflammation scores. Assessment of treatment responses in r-axSpA patients refractory to anti-TNF demonstrated higher ASAS40 responses in the ustekinumab 45mg (19%) and 90mg (27%) groups compared to the placebo group (12%) but early discontinuation of this RCT with only 44% of the planned sample size precluded valid statistical analysis and clinical conclusions. Moreover, separation from placebo was not consistent across endpoints and there was no benefit of ustekinumab in reducing CRP in this RCT. Frequencies of adverse events were comparable among treatment groups with no new safety signals. Of particular interest, neither T_H17 cytokines (IL-17A, IL-17F, IL-22, and IL-23) nor T_H1 cytokines (IFNγ and IL-12p70) were dysregulated at baseline in AS patients compared with healthy controls. Moreover, ustekinumab had only a minor impact on biomarkers at weeks 4 and 16 with only MMP3, serum amyloid A (SAA), and IL-8 being significantly decreased, and this change did not correlate with clinical response.

These RCTs conclusively demonstrate a lack of impact of targeting IL-23 for alleviation of inflammation in axSpA according to both patient self-reported outcomes, objective measures of inflammation, and biomarkers reflecting inflammation and tissue turnover. These RCTs also highlight the limitations of open label studies in a disease where the primary outcomes assessed in clinical trials are patient self-reported assessments of symptoms such as pain, stiffness, and global well-being. The open label study of ustekinumab did report an overall reduction of 30-40% in MRI scores for inflammation in the SIJ and spine after 24 weeks but this is substantially less than the 60-70% reductions noted in trials of TNFi agents. Moreover, it has been shown that fluctuation over a 3-month time frame in MRI inflammation in the SIJ beyond measurement error may be observed in about 20% of patients despite stable NSAID and/or DMARD intake (91). Consequently, it is very important to design a clinical trial program for novel therapeutics in axSpA that includes an appropriately powered phase II placebo-controlled RCT with MRI inflammation as an endpoint since demonstration of significant treatment group differences in MRI scores has been a consistent indicator of an efficacious therapeutic.

Guselkumab

A recent report has added yet another twist to the potential therapeutic role of agents targeting IL-23 (92). The efficacy of guselkumab (GUS), a human monoclonal IgG1λ antibody that selectively binds to the p19 subunit of IL-23, was examined in patients with back pain in a post-hoc analysis of two phase III placebo-controlled RCTs that evaluated this agent in active peripheral PsA (93). In DISCOVER-1, 381 pts with active PsA (≥ 3 swollen joints, ≥ 3 tender joints; C-reactive protein ≥ 0.3mg/dL despite standard therapies) and in DISCOVER-2, 739 pts with active PsA (≥ 5 swollen joints, ≥ 5 tender joints, CRP ≥ 0.6mg/dL despite standard therapies) were randomized 1:1:1 to GUS 100mg Q4W, GUS 100mg Q8W (Wk0, Wk4, then Q8W), or placebo. This analysis included patients with sacroiliitis at baseline who had either documented imaging confirmation of sacroiliitis in the past or pelvic X-ray confirmation of sacroiliitis at screening (pooled data from DISCOVER-1&2) based on local investigators' judgment of presence/absence of sacroiliitis. Central reading of imaging was not performed. Efficacy was assessed by BASDAI score, BASDAI50 response, spinal pain, ASDAS-ID, and ASDAS-MI. There were 312 patients with axial involvement and those in the GUS arms had significantly greater clinical responses with BASDAI50 improvement noted in 40.5% and 37.9% of the 100mg Q8W and 100mg Q4W dosing arms, respectively, as compared to 19.1% of those on placebo. A major challenge in the interpretation of this data is whether the clinical responses truly reflect improvement in axial *versus* peripheral inflammation as impact of treatment on objective features of inflammation, especially MRI of the SIJ and spine, was not reported. A second major challenge is the lack of a case definition for psoriatic axial involvement so that appropriate patients can be selected for clinical trials. Again, this will most likely require evidence of both active and structural lesions on MRI of the SIJ and spine.

Beyond the trial design issues outlined in the preceding paragraphs, the recent discovery of IL-17 production independent of IL-23 could be the explanation for the lack of success of IL-23 blockade in axSpA [ref]. Although IL-23 and IL-17 are closely related and form a classically described "IL-23/IL-17 pathway", cells other than T_H17 cells can be the source of IL-17, including MAIT cells, γδ T cells, NK cells and ILCs (**Figure 1**). These other RORγt+, Type 17 cells could express IL-17 upon stimulation by cytokines other than IL-23, and therefore, blocking IL-23/IL-23R would not be sufficient to suppress the downstream effects of IL-17. The co-existence of IL-23 dependent and independent IL-17 production has been supported by laboratory data both from human tissues and from animal models. In human entheseal tissue, two subsets of γδ T cells were identified, Vδ2 subset cells that express IL-23-inducible IL-17 associated transcripts, and Vδ1 subset cells that completely lack detectable IL-23R but express IL-17 transcript upon stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin, suggesting the presence of IL-23 independent expression of IL-17 in human entheseal tissue (94) (**Figure 3**). Consistent with this finding and the importance of IL23-independent regulation of IL-17 in human disease, patients with AS have a higher frequency of IL-17A positive MAIT cells in peripheral blood and in synovial fluid (95). IL-7, but not IL-23, induces IL-17A expression in these MAIT cells (95).

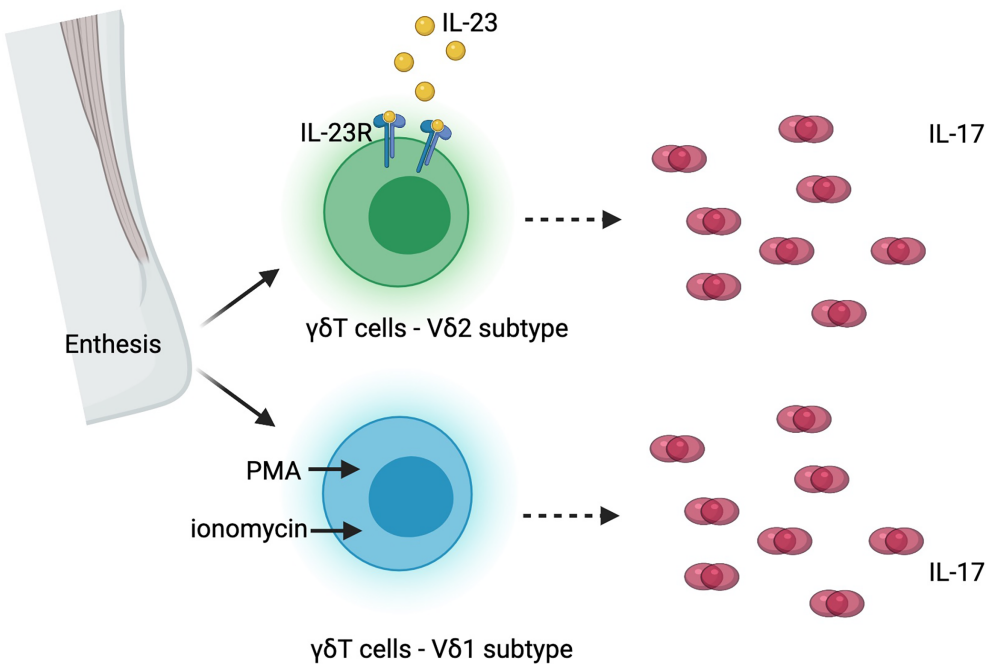

FIGURE 3 | Co-existence of interleukin-23 (IL-23) dependent and independent interleukin-17 (IL-17) production in human enthesis. PMA, phorbol 12-myristate 13-acetate; IL-23R, interleukin-23 receptor.

Inhibition of IL-23 in collagen-induced arthritis and SKG mice ameliorated inflammatory arthritis but did not completely abolish it (96, 97). Targeting IL-23R in the HLA-B27 transgenic rat, an experimental model that closely resembles human SpA, was not effective once arthritis was established but did have some beneficial impact in preventing the onset of disease, indicating IL-23 independent production of IL-17 in the perpetuation of disease (38). All these data suggest IL-17 can be induced by other cytokines in non-T_H17 cells, and there could be a co-existence of IL-23 dependent and IL-23 independent IL-17 expression in entheseal tissues (**Figure 3**).

IL-17 EFFECT ON BONE METABOLISM

Inflammation related bone loss and osteoporosis are often seen in patients with inflammatory arthritis, including patients with AS. Paradoxically, the hallmark of AS is new bone formation at the entheseal sites, particularly syndesmophyte formation and ankylosis of the spine. The mechanism of co-existence of new bone formation at entheseal sites and diffuse bone loss is not fully understood.

IL-17 and Osteoclastogenesis

IL-17 has long been reported to play an important role in bone loss, by either inducing osteoclastogenesis or by suppressing Wnt signaling. Its effects likely depend on the type of cell and its stage of differentiation as well as the cellular composition and the extracellular milieu, especially the levels of other cytokines and inflammatory mediators. It may impact various cells mediating cartilage and bone destruction, but it may also act on mesenchymal cells and osteoblasts. Co-culture of T_H17 cells and monocytes induced osteoclastogenesis in mice, and this process is possibly also mediated by IL-17 (98). In a coculture system of murine hematopoietic cells and primary osteoblasts, IL-17 induced expression of RANKL in osteoblasts, which in turn, induced differentiation of osteoclast progenitors into mature osteoclasts (99). In a mouse model of IL-17A mediated skin inflammation, keratinocytes, γδ T cells, and ILCs expressed IL-17A, and inhibited osteoblast and osteocyte function *via* inhibition of Wnt signaling (100). Consistent with this finding, more periosteal bone formation was observed at peak inflammation in IL-17A deficient mice (101). Furthermore, *in vitro* study showed that IL-17A inhibited osteoblast differentiation by inducing mRNA expression of the Wnt signaling antagonist secreted frizzled related protein-1 (sFRP1) and suppressing mRNA expression of sFRP3, which has been shown to promote osteoblast differentiation in bone marrow stromal cells (101). IL-17A might also have a direct effect on osteoclasts. In an *in vitro* study of human CD14+ monocytes, IL-17A upregulated RANK expression in osteoclast progenitors and therefore could directly induce osteoclastogenesis (102).

IL-17 and Osteoblastogenesis

Other studies, which include murine fracture healing models and evaluation of human mesenchymal stem cells, suggest that IL-17 promotes osteoblastogenesis and new bone growth. In a murine fracture model, IL-17F was reported to induce osteoblast maturation and mediate the early phase of fracture repair (103). A further study in mice with bone injury demonstrated that IL-17A was produced immediately after injury by Vδ6+ γδ T cells in the peripheral tissues, and promoted osteoblastogenesis by increased proliferation of mesenchymal cells (104). In humans, activated T cells or derived condition medium triggers osteogenic differentiation in bone marrow derived mesenchymal stem cells, and this effect was mediated by IL-17A and IL-17F (105). In mesenchymal stem cells isolated from human spinal peri-entheseal bone, IL-17A enhances osteogenesis and suppresses adipogenesis (106).

The effect other of cytokines involved in IL-23/IL-17 pathway, in addition to IL-17, on bone formation and bone reabsorption has been reviewed elsewhere (107).

EFFECT OF IL-17 INHIBITION IN PATIENTS WITH ANKYLOSING SPONDYLITIS

In axSpA, preclinical data suggested that IL-17 may play a role in osteogenesis and that inhibition of IL-17 might be associated with less new bone formation. An *in vitro* study showed that IL-17A promotes osteogenesis in synovial fibroblasts from patients with active axSpA (39). Serum from patients with AS can induce osteogenesis in human periosteum-derived cells *in vitro*, and this effect can be blocked by bimekizumab, the dual inhibitor of IL-17A and IL-17F (19). Further, in the HLA-B27 transgenic rat model, administration of an IL-17A inhibitor antibody after disease onset resulted in new bone formation in 9 vertebrae from 3 rats, while treatment with control IgG2a resulted in new bone formation in 11 vertebrae from 5 rats, a numerical decrease but not statistically significant (39). It has been shown that circulating IL-17 increases with the onset of ankylosis in male DBA/1 mice, who develop enthesitis after being caged together, which then proceeds to ankylosis (108). Prophylactic administration of anti-IL-17 antibodies significantly prevented the development of ankylosis while administration after disease onset ameliorated but did not completely prevent ankylosis.

Clinical data for radiographic progression in patients with AS treated with IL-17A inhibitors is scant and inconclusive. Recent studies have used the modified Stoke AS Spine Score (50) (mSASSS, see **Box 1**) to quantify new syndesmophytes and radiographic progression in AS. In patients treated with SEC 150mg Q4W (n=86) *versus* 75mg Q4W (n=82) for 2 years, a comparable increase in mSASSS (0.30 +/- 1.94 in the 150mg dose group *vs.* 0.31 +/- 3.04 in the 75mg dose group) was observed (109). After 4-year treatment, the mean change in mSASSS was 1.2 +/- 3.91 in the 150mg dose group (N = 71) *vs.* 1.6 +/- 5.67 in the 75mg/up titration dose group (up titrate to 150mg, N = 23) *vs.* 1.8 +/- 4.32 75mg dose group (N = 61) (110), without a significant dose effect. When compared to a historical cohort of biologic naïve patients treated only with NSAIDs over 2 years,

the change in mSASSS in SEC treated patients was 0.55 +/- 0.139 (N = 168), numerically lower than the change of mSASSS in the historical cohort (0.89 +/- 0.216, N = 69) (111). The proportion of patients without radiographic progression was also higher in the SEC group than historical cohort, but again, not statistically significant (mSASSS change <= 0: 60.7% in SEC group *versus* 52.2% in historical cohort; mSASSS change <= 2: 82.1.7% in SEC group *versus* 72.5% in historical cohort) (111).

However, the interpretation of these clinical data is limited by the reliability and sensitivity to change of the mSASSS and the absence of a control group which leads to a conservative bias to scoring change. This radiographic scoring method records radiographic features (sclerosis, squaring, erosion, syndesmophyte, ankylosis) in the anterior vertebral corners on lateral projections of cervical and lumbar spine radiographs, with a total possible score of 0-72 (50). In biologic naïve patients, 30 - 40% demonstrate an increase in mSASSS over 2 years, with an average change of 1.0 +/- 2.9 units (112). When used in clinical trials to assess the efficacy of a given treatment over placebo, a sample size of 100 in each arm would be needed to detect a difference between arms over 2 years (113). In addition, the inter-reader reliability was only 54% when assessing progression over 2 years (112). Consequently, the limited sample size in the above studies and measurement error preclude the demonstration of a statistically significant result for IL-17 inhibition on radiographic progression. Evidence of a lag effect of TNFi therapies on radiographic progression strongly suggests that at least 4-year follow up will be desirable which will necessitate active comparator studies as placebo-controlled trials will not be feasible. As yet, there are no soluble biomarkers that reflect radiographic progression which can be targeted in clinical trials but increasing evidence suggests that MRI inflammation in the spine may be a valid and more responsive surrogate.

CONCLUSIONS

It has been a long way from the discovery of IL-23/IL-17 pathway to its clinical application in the treatment of patients with axSpA and SpA related conditions. While the inhibition of IL-17 cytokines has been a great success in controlling inflammatory symptoms in patients with axSpA, IL-23 blockade was not effective in treating axSpA. Despite that IL-17 inhibition is not effective and even harmful in treating IBD, but it does not seem to increase the incidence of IBD when given to patients with axSpA. It also seems ineffective for uveitis. These findings at the bedside brought new insights at the bench, suggesting tissue heterogeneity of expression and function of IL-23 and IL-17 cytokines. In addition to the classically described IL-23/IL-17 pathway, cells other than T_H17 cells could express IL-17 in an IL-23 independent fashion. Laboratory evidence has suggested a co-existence of IL-23 dependent and independent IL-17 production in human entheseal tissues. Future work should focus on how to dissect these two processes at a clinical level in patients with axSpA, using objective measures for inflammation, such as CRP, MRI, and innovative biomarkers. Whether IL-17 inhibition will impede radiographic progression in patients with axSpA remains inconclusive. Future work should focus on improving study design, outcome measures, and identifying imaging and soluble biomarkers for disease progression.

AUTHOR CONTRIBUTIONS

RW and WM contributed to conception of the review, and conducted literature review. RW and WM wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

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Paradoxical Augmentation of Experimental Spondyloarthritis by RORC Inhibition in HLA-B27 Transgenic Rats

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Objective: IL-17A plays a major role in the pathogenesis of spondyloarthritis (SpA). Here we assessed the impact of inhibition of RAR related orphan receptor-γ (RORC), the key transcription factor controlling IL-17 production, on experimental SpA in HLA-B27 transgenic (tg) rats.

Methods: Experimental SpA was induced by immunization of HLA-B27 tg rats with heat-inactivated *Mycobacterium tuberculosis*. Splenocytes obtained at day 7, 14 and 21 after immunization were restimulated *ex vivo* to assess the induction of pro-inflammatory cytokines. Rats were then prophylactically treated with a RORC inhibitor *versus* vehicle control. The biologic effect of RORC inhibition was assessed by pro-inflammatory cytokine expression in draining lymph nodes. Arthritis and spondylitis were monitored clinically, and the degree of peripheral and axial inflammation, destruction and new bone formation was confirmed by histology.

Results: *Ex vivo* mRNA and protein analyses revealed the rapid and selective induction of IL-17A and IL-22 production by a variety of lymphocyte subsets upon disease induction in HLA-B27 tg rats. Prophylactic RORC inhibition *in vivo* suppressed the expression of IL-17A, IL17F, and IL-22 without affecting the expression of other T helper cell subset related genes. This biological effect did not translate into clinical efficacy as RORC inhibition significantly accelerated the onset of arthritis and spondylitis, and aggravated the clinical severity of arthritis. This worsening of experimental SpA was confirmed by histopathological demonstration of increased inflammation, destruction, and new bone formation.

Conclusion: Despite a significant suppression of the IL-17 axis, RORC inhibitor treatment accelerates and aggravates experimental SpA in the HLA-B27 tg rat model.

Keywords: spondyloarthritis, IL-17A, RORC, IL-22, HLA-B27 transgenic rats

INTRODUCTION

The IL-17 axis plays an important role in the immunopathology of spondyloarthritis (SpA) as indicated by a wealth of genetic, immunopathological, experimental, and translational evidence (1). Randomized clinical trials have formally demonstrated the role of IL-17A, the prototypical IL-17 cytokine, in human SpA. Treatment with Secukinumab (anti-IL-17A mAb) reduced signs and symptoms of ankylosing spondylitis (AS) as well as of psoriatic arthritis (PsA), the major forms of axial and peripheral SpA, respectively (2, 3). Similarly, ixekizumab has proven clinical efficacy in PsA (4) and AS (4). Recent evidence, however, suggests that other cytokines of the IL-17A family, with as prime example IL-17F, can contribute to inflammation in SpA (5). Albeit not proven clinically, animal models have also suggested a potential role for IL-22 (6, 7). This raises the question whether targeting other molecules in the IL-17 axis could enhance clinical efficacy above and beyond selective IL-17A blockade.

One of the key molecules controlling the production of IL-17A and related cytokines is the transcription factor retinoic acid receptor-related orphan receptor RORγt (murine) or RORC (the human homologue). RORC is involved in the production and regulation of IL-17A by different cell types including Th17 cells (8), γδ T cells (9), innate lymphoid cells (10). In animal models a distinctive group of RORγt+ Tregs that is vital to maintain gastrointestinal homeostasis and avoid colitis (11–13). Moreover, it is crucial in orchestrating the differentiation of naïve CD4 T cells to Th17 cells (8). Enhanced gene transcription of RORC increases IL-17A production in a T cell line and human primary cells (14–16), whereas blockade of RORC in human CD4 T cells suppresses IL-17A and other inflammatory cytokines, including IL-17F and IL-22 (17, 18). Expression of IL-23R, CCR6, and IL-26 were also decreased upon RORC inhibition in Th17 cells, without affecting the gene signature of other T helper cell types (18, 19). Genetic lack of RORγt protected mice against experimental autoimmune encephalomyelitis (EAE), induced defects in Th17 differentiation and prevented T-cell-transfer-mediated colitis (8, 20). Pre-clinical studies in animal models, including imiquimod-induced psoriasis (21), spontaneous colonic inflammation (22), antigen induced arthritis (17), EAE (23), and experimental autoimmune uveitis (EAU) (24), confirmed that RORC inhibition markedly reduces local and systemic IL-17A levels and decreases tissue inflammation. Moreover, expression of IL-17F and IL-22 was also reduced upon *in vivo* RORC inhibition (21, 23). These findings indicate that RORC could be an interesting therapeutic target in IL-17A dependent pathology, blocking not only IL-17A but also related pro-inflammatory cytokines.

In this study, we aimed to assess RORC as potential therapeutic target in SpA by studying the effects of a small molecule RORC inhibitor in experimental SpA in HLA-B27 transgenic (tg) rats, a well validated model for human SpA (25–27). As we previously showed that both initiation and disease persistence in this model is partially but not completely inhibited by IL-17A blockade (28), we used this model to assess how RORC inhibition affects a panel of IL-17 related cytokines *in vivo* and if this biological effect translates into clinical efficacy.

METHODS

Animals

The inducible HLA-B27 tg x beta-2 microglobulin (B2M) tg rat model has been described previously (27). Briefly, the Tg (HLA-B*2705,B2M) 21-3Reh and Tg(B2M) 283-2Reh rat lines (25) on Lewis background were bred and housed (3 per cage) in individually ventilated cages at the animal research institute AMC. Six weeks-old F1[21-3x283-2] animals were immunized with low dose (30-90 µg depending on sex and housing conditions) heat-inactivated *Mycobacterium tuberculosis* (MTB) (Difco, Detroit, MI) in 100 µl Incomplete Freund's Adjuvant (IFA) (Chondrex) *via* subcutaneous injection in the tail base. The immunization strategy using heat-inactivated *Mycobacterium tuberculosis* as a broad innate immune receptor trigger. In the HLA-B27 tg rats, immunization using 30–60 µg *M. tuberculosis* was sufficient to induce spondylitis and arthritis in both male and female rats (80-100%). Without immunization spontaneous development of spondylitis and arthritis appeared in 40% of the male rats around 9 months of age in the presence of a severe autoimmune epididymoorchitis which is clinically manifested in 100% of the male rats at 3 months of age. Clinical disease (arthritis and spondylitis) appears 20-30 days after immunization. All animal experiments were approved by the AMC Animal Care and Use Committee, in line with national and international regulations and guidelines. The data presented are the data of two separated experiments combined.

Ex Vivo Restimulation Experiments

Mononuclear cells from spleen were isolated from F1[21-3x283-2] rats at day 7, 14, 21 after immunization with MTB in IFA (n=4/time point). At each time point two non-immunized rats matched by age and gender, were taken along as control. The non-immunized rats (n=6) were pooled for all analyses. Cells were used either unstimulated or after *ex vivo* restimulation with 10 ng/ml PMA and 1 µg/ml ionomycin for 6 hours. Using splenocytes, mRNA expression was measured using Taqman assays (Thermo Fisher Scientific) for *il17a* (assay ID Rn01757168_m1), *tnf* (assay ID Rn99999017_m1) and *ifng* (assay ID Rn00594078_m1) with *gapdh* (assay ID Rn01775763_g1) as housekeeping gene. Data were analyzed according to the $2^{-\Delta\Delta CT}$ method (29). IL-17A, TNF, and IFNy protein secretion was measured by ELISAs in the culture supernatant stimulated for 24 hours (Thermo Fisher Scientific IL-17A ELISA kit #88-7170-88; R&D duoset IFNy ELISA #DY-585). Intracellular protein expression of IL-17A and IFNy (*versus* isotype control) by different lymphocyte subsets was assessed by FACS in splenocytes stimulated overnight (in the presence of 10 µg/ml Brefeldin for the final 4 hours). Data was recorded using a FACS Canto II and analyzed using FlowJo software.

In Vivo RORC Inhibition

The RORC inhibitor BI119 (Boehringer-Ingelheim Pharmaceuticals Inc., Ridgefield, CT, USA) was discovered by screening a small-molecule compound library. BI119 strongly bound to the human RORyt ligand-binding domain (LBD) and was active in an RORyt LBD reporter assay (Kd for RORy LBD – 65 nM; IC50 for RORy LBD reporter assay 260 nM). The compound showed high selectivity towards RORyt as demonstrated by a lack of significant activity against RORa (IC50 > 10 μM) and RORb (IC50 > 6 μM). Rats (n=6/group) received 30 mg/kg RORC inhibitor dissolved in Natrosol™ 250 Hydroxyethylcellulose (further referred to as Natrosol) (Ashland Specialty Ingredients #88-7170-88) or Natrosol alone twice daily *via* oral gavage. Assignment to treatment was random and per cage to avoid cross contamination *via* feces. Treatment started one week after immunization and continued for five weeks. Experiment was done twice with 6 rats/group.

Serum Exposure Measurement

Serum samples were collected *via* Saphenous vein puncture, 2 hours after the morning dose at day 31 and before the morning dose at day 32 post immunization. The concentration of BI114 was determined by liquid chromatography-mass spectrometry analysis.

Downstream Cytokine Analyses

After *in vivo* treatment, RNA was isolated from popliteal lymph nodes using TRIzol. Samples were analyzed with a rat Th17 qPCR array according to the manufacturers protocol (Qiagen # PARN-073Z). Selective genes were confirmed by regular qPCR with SYBR green primers for IL-17A, IL-22, IL-17F, IL-13 and GAPDH as housekeeping gene (all primer sequences are available upon request). Data were analyzed according to the $2^{-\Delta\Delta CT}$ method (29).

Clinical Scoring of Arthritis and Spondylitis

The presence of arthritis in the paws was determined clinically and digital hind paw swelling was measured with plethysmometry. Arthritis severity in each paw was graded 0-3 as described before (27). Cumulative clinical scores were calculated for severity analysis. Swelling in cm^3 was normalized to the days before disease onset. Spondylitis was determined clinically by swelling and bumps in the tail and scored yes/no. In case of humane endpoints, due to ethical considerations, rats were sacrificed with the last observation carried forward. Humane endpoints were defined as 15% bodyweight loss or two completely swollen paws. One rat in the vehicle treated group was sacrificed due to reaching the humane endpoint for bodyweight loss. Clinical scoring was performed by one observer, blinded for treatment.

Histology

Hind paws and tails were decalcified in Osteosoft (Merck) and embedded in paraffin. Sections were stained for hematoxylin and eosin or safranin O and semi-quantitatively scored by two observers blinded for treatment (MT, LD) as previously described (26).

Statistics

Data were analyzed using GraphPad prism 7 software. Spondylitis and arthritis incidence were analyzed using a survival curve. Comparison of survival curves was analyzed using the Log-Rank (Mantel-Cox) test. Arthritis severity (arthritis score and hind paw swelling) was analyzed using the area under the curve followed by a Mann-Whitney U test. All other data were analyzed using a Mann-Whitney U test.

RESULTS

Induction of Experimental SpA in HLA-B27 Tg Rats Is Associated With a Rapid and Selective Induction of IL-17A and IL-22

We previously demonstrated that selective blockade of IL-17A significantly reduces spondylitis and arthritis development in the inducible HLA-B27 tg rat model of SpA (28). To further assess the potential involvement of IL-17A and related pro-inflammatory cytokines, *ex vivo* cellular responses to MTB immunization were assessed. To this end cytokine expression and production were determined in splenocytes upon restimulation with PMA/Ionomycin at 7, 14 and 21 days after MTB immunization. RNA expression analysis indicated a trend towards an increase in IL-17A, but not in IL-17F expression upon restimulation, with the peak-response at 7 days after immunization. Furthermore IL-22 was increased upon restimulation at 7 days after immunization. Other pro-inflammatory cytokines including TNF and IFNy as well as the expression of RORC, were not changed after immunization (**Figure 1A**). Protein secretion analysis confirmed the trend towards increased levels of IL-17A at 7 days after immunization (**Figure 1B**), while secretion of TNF and IFNy was not detectable at any time point upon restimulation with PMA/Ionomycin. A repetitive experiment with samples collected at day 7 (n=2 non-immunized and n=3 immunized), confirmed the increase in IL-17A expression, and the unchanged expression of IFNy upon immunization. To assess which cells were responsible for IL-17A production in our model FACS analysis was performed, focusing on three T cell subsets: CD4+, CD4- and γδ T cells. The increased presence of IL-17A$^+$ cells at 7 days after immunization could be confirmed, within the population of CD4+ TCRab$^+$, CD4-TCRab$^+$ and CD4-TCR γδ$^+$ T cells (**Figure 1C**). The frequency of IFNy$^+$ cells was low in all subsets, with no differences between the different time points (**Figure 1C**). Collectively, these data indicate the rapid and selective induction of IL-17A production by a variety T cell subsets upon MTB immunization in HLA-B27 tg rats.

In Vivo RORC Inhibition Suppresses IL-17A, IL-17F and IL-22 Expression in HLA-B27 Tg Rats

To assess the potential relevance of RORC - the key transcriptional regulator of IL-17A and related cytokines - as therapeutic target, we performed an *in vivo* prophylactic treatment study with RORC inhibition *versus* vehicle control in our HLA-B27 tg at model (**Figure 2A**). Serum measurements

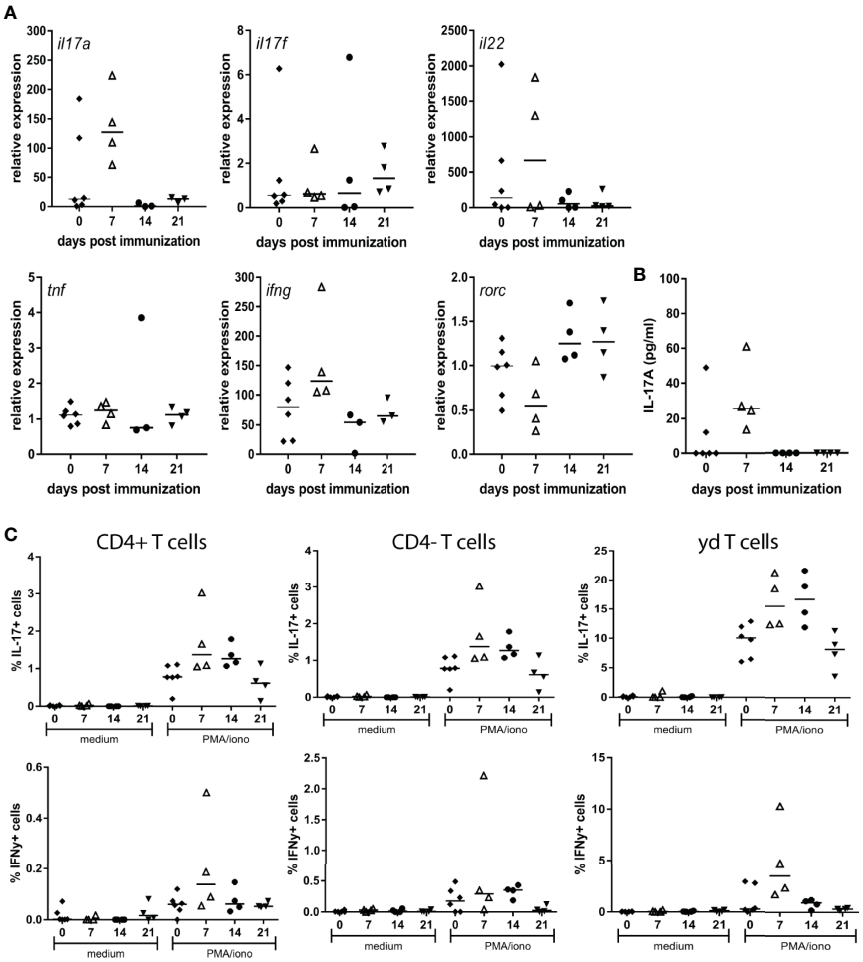

FIGURE 1 | Restimulation of splenocytes from MTB immunized HLA-B27 tg rats primarily induced IL-17A. **(A)** Relative expression of il17a, il17f, il22, tnf, ifng and rorc **(B)** protein secretion of IL-17A **(C)** FACS analysis of IL-17A and IFNγ expressing T cell subsets.

confirmed that all rats in the treatment group, but not in the vehicle control group, had high exposure to the compound (**Table 1**). Whereas a variety of previous experiments with human/mouse cells indicated an IC50 of 10 to 20 nM (data not shown), we confirmed that the exposure levels seen *in vivo* in the rats significantly inhibited IL-17A production by γδ T cells of both wildtype and HLA-B27 tg rats *in vitro* (**Figure 2B**).

We next investigated whether the expression of IL-17A and related cytokines was also reduced *in vivo* by RORC inhibition by analyzing popliteal lymph nodes obtained at the end of the *in vivo* study. IL-17A, IL-17F, and IL-22 expression was more than 2-fold downregulated as measured in a qPCR array (**Figure 2C**). These data were confirmed by regular qPCR (**Figure 2D**). The Th2 cytokine IL-13 initially showed a tendency towards an increase upon RORC inhibition in the arrays (**Figure 2C**) but this could not be confirmed by regular qPCR (**Figure 2D**). Expression of a wide panel of Th1, Th2, and Treg cytokines or transcription factors, or other genes included in the Th17 array, was not different between vehicle treated and RORCi treated rats. Together these data indicate that RORC inhibition *in vivo* suppressed the expression of IL-17A, IL17F, and IL-22 without affecting the expression of other T helper cell subset related genes.

In Vivo RORC Inhibition Augments Experimental SpA in HLA-B27 Tg Rats

To assess if the observed biological effects on the IL-17 axis translate into clinical efficacy, clinical disease development was monitored over time and histopathological analysis was performed at the end of the study. All RORC inhibitor-treated rats developed both spondylitis and arthritis *versus* 70% and 100%, respectively, in the vehicle-treated group (**Figure 3A**). The mean onset of spondylitis was day 25 in RORC inhibitor *versus* day 34 in vehicle treated rats. The mean onset of arthritis was day 21 in RORC inhibitor *vs* day 33 in vehicle treated rats. In terms of arthritis severity, both arthritis score (p=0.004) and hind paw swelling as assessed by plethysmometry (p=0.001) were increased upon RORC inhibition (**Figure 3B**). Histopathological analysis of the peripheral joints at the end of the experiment confirmed a

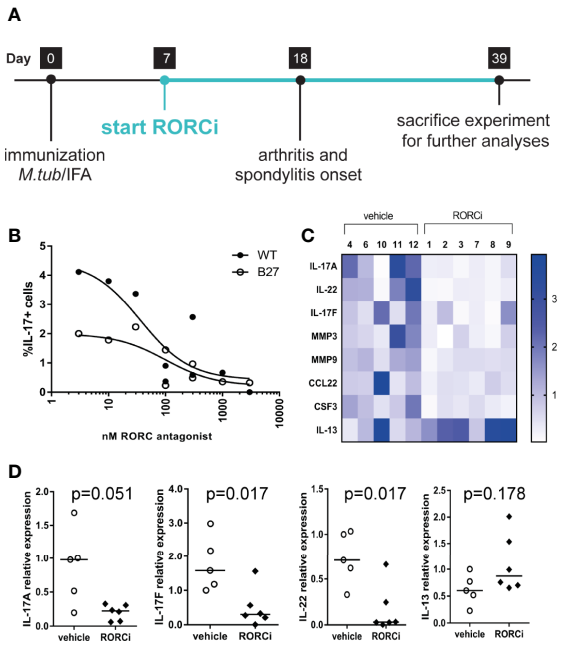

FIGURE 2 | *In vivo* and *in vitro* inhibition of RORC. **(A)** Study design of *in vivo* prophylactic treatment **(B)** Dose response effect of *in vitro* RORC inhibition on frequencies of IL-17+ yd T cells. **(C)** Relative expression of genes >2fold differentially regulated in vehicle *vs* RORCi treated rats. **(D)** Gene expression from the selected genes, IL-17A, IL-22, IL-17F and IL-13 was confirmed by regular qPCR. Data are individual data points and median.

significant increase in inflammatory infiltration (p=0.004), destruction (p=0.003), newly formed bone (p=0.001), and hypertrophic chondrocytes (p=0.001) in RORC inhibitor treated rats compared to the controls (**Figure 4**). A similar trend towards increased inflammation (p=0.058) and bone destruction (p=0.061) was observed in the spine (**Figure 4**). Collectively, these data consistently demonstrate that, despite the expected biological impact of RORC inhibition on the IL-17 axis, this treatment did not inhibit but rather accelerated and aggravated experimental SpA in HLA-B27 tg rats. To investigate if RORgt inhibition affected the gut we performed histology on the colon and small intestine. No inflammation was observed in the colon and the small intestine. Combined with the absence of weight loss, these data show that the aggravation of the SpA phenotype cannot be explained by triggering of disease by RORgt inhibition induced gut inflammation.

TABLE 1 | Serum levels of RORC inhibitor.

Rat	Treatment	Concentration (nM)	
		before	after
1	RORCi	2491	2055
2	RORCi	1487	1576
3	RORCi	1425	1873
4	Vehicle	0	0
5	Vehicle	0	0
6	Vehicle	0	0
7	RORCi	3527	4691
8	RORCi	1187	4673
9	RORCi	1891	1762
10	Vehicle	0	0
11	Vehicle	0	0
12	Vehicle	0	0

RORCi serum exposure was measured in rats of both groups (RORCi vs vehicle) before the morning dose and 2-3 hours after the morning dose at 1 timepoint only: day 32/33 of treatment.

DISCUSSION

The major findings of the current study are 1) that IL-17A is primarily produced upon restimulation of splenocytes from MTB immunized rats, with a peak response after 7 days of immunization. 2) IL-17A expression could be reduced upon *in vitro* as well as *in vivo* RORC inhibition, additionally *in vivo* RORC inhibition reduced IL-17F and IL-22 expression without affecting other T helper cell related genes. 3) Despite these molecular changes RORC inhibition did not inhibit, but rather stimulated disease development. These findings are surprising considering previous studies of RORC inhibition in a variety of inflammatory models including antigen-induced arthritis (17), imiquimod-induced psoriasis (21), IL-23 induced skin inflammation (30), intestinal inflammation (22, 31). Recently, Tan et al. (24) showed the reduced clinical severity of experimental autoimmune uveitis (EAU), and EAE using two different RORc antagonists (CQMU151 and CQMU152) (24).

All these models showed decreased levels of IL-17A and significant reduction of clinical symptoms upon pharmacological RORC inhibition or in RORC deficient animals. Also in 2018, Taurog et al. demonstrated the therapeutic efficicacy of the RORγt antagonist (A-1619758) in reducing inflammation, and suppression of both axial and peripheral skeletal bone changes in the HLA-B27 transgenic rat deficient for *Dazl gene* (21-3x283-2x17-9) (32).

A major question is whether the data of the current study could have been biased by a technical or biological artefact. Exposure measurements indicated that all rats in the treatment group have high levels of RORC inhibitor present in their serum shortly before and a few hours after treatment. The control group responded as expected, although disease incidence and severity were low. We could detect a biological effect, in terms of reduced IL-17A, IL-17F and IL-22 expression and finally clinical data are in line with histological data. Together these findings prove that groups were not switched during or after this experiment. The potential mechanism remains unknown.

Others showed, using the exact same RORC inhibitor, that while IL-17A levels were reduced, the production of IL-22 continued (33, 34) and treatment with the RORC inhibitor abrogated experimental colitis (34). Specific subsets of iNKT and γδ T cells showed to be among the IL-22 producing cells upon RORC inhibition (33). Similarly another RORC inhibiting compound could selectively impact specific cell types, including Th17 cells, while local IL-17A or IL-22 production by ILC3s was not reduced in a mouse model for intestinal inflammation (22). IL-22 has been shown to be a key player in intestinal host defence and mucosal homeostasis (35), and as we did observe reduced

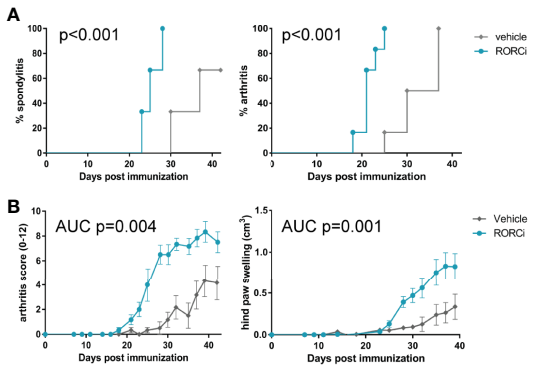

FIGURE 3 | RORC antagonist accelerated spondylitis and arthritis development and increased arthritis severity. **(A)** Incidence of spondylitis and arthritis in vehicle *vs* RORCi treated rats **(B)** disease severity, displayed as arthritis score and hind paw swelling in vehicle *vs* RORCi treated rats. Data are mean +/- SEM.

IL-22 expression, it could by hypothesized that RORgt inhibition could result in gut inflammation by a decrease in IL-22. This gut inflammation could then induce the SpA phenotype in the rats, similar to the induction of disease by orchitis, which is a consistent finding in this animal model. However, the rats showed no weight loss (**Figures 5A, B**) and gut inflammation was absent after RORgt inhibition, which makes this hypothesis very unlikely.

In contrast, in another model for intestinal inflammation the RORgt inverse agonist (TAK-828F) suppressed intestinal inflammation. In this model the compound significantly reduced Th17 and Th17/Th1 cell population in mesenteric lymph nodes (MLN) which was accompanied by suppressed/decreased gene expression of not only IL17A, IL17F but also IL-22. Local effects on ILC3 function in the intestine was not studied. However, in normal mice this RORgt inverse agonist did reduce numbers of Th17 cells and IL3s in the laminal propria (31).

In order to test the responsiveness of cells in the MTB immunized HLA-B27 tg rat $CD4^+$ T cells and γδ T cells were isolated from spleen and draining lymph nodes and restimulated in the presence of RORC inhibitor (n=9). IL-17A and IL-22 expression were similarly decreased in both cell subsets upon RORC inhibition.

It is important to note, however, that this is not the first time that clinical or preclinical data demonstrate that the IL-17 axis does not work in a linear manner. It was reported by Chong et al., that loss of IL-17A produced by Th17 cells didn't reduce the pathogenicity of these cells as expected and instead increased the expression of other Th17 cytokines (i.e. GM-CSF and IL-17F) (36). This was attributed to a Th17 cell-intrinsic autocrine loop induced by IL-17A binding to its receptor resulting in IL-24 induction, which in turn repressed the Th17 cytokine program. They showed that *in vivo* IL-24 treatment ameliorated Th17-induced EAU, whereas silencing of IL-24 in Th17 cells enhanced disease. However, these findings are unlikely to explain the paradoxical effects of RORC inhibition in our model as we observed the down regulation of other TH17 cytokines (e.g. IL-17F).

Clinical studies in psoriasis patients indicated that both blockade of IL-23 as well as IL-17A are effective (37, 38). However in Crohns disease blockade of IL-23 was effective while blockade of IL-17A or IL-17RA was not (39–41). Finally in SpA, blockade of IL-23p19 seems to be effective in psoriatic arthritis (42) but not in ankylosing spondylitis (43). Similarly in a previous study with our HLA-B27 tg rats, we showed that blockade of the IL-23 receptor (IL-23R) significantly reduced levels of IL-17A and IL-22 (but not IL-17F) *in vivo*. While prophylactic blockade of the IL-23R completely prevented spondylitis and arthritis development, therapeutic blockade did not impact clinical or histological spondyloarthritis in the HLA-B27 tg rat (44). Moreover prophylactic as well as therapeutic blockade of IL-17A was effective in this model (28).

The RORγt+ Tregs subset might be involved in these unanticipated effects. The function of these regulatory cells was thought to be controlled partly by RORγt (45). Kleinewietfeld et al. showed that a low molecular weight RORγt inhibitor could influence the frequencies of regulatory T-cells (Tregs) beside the

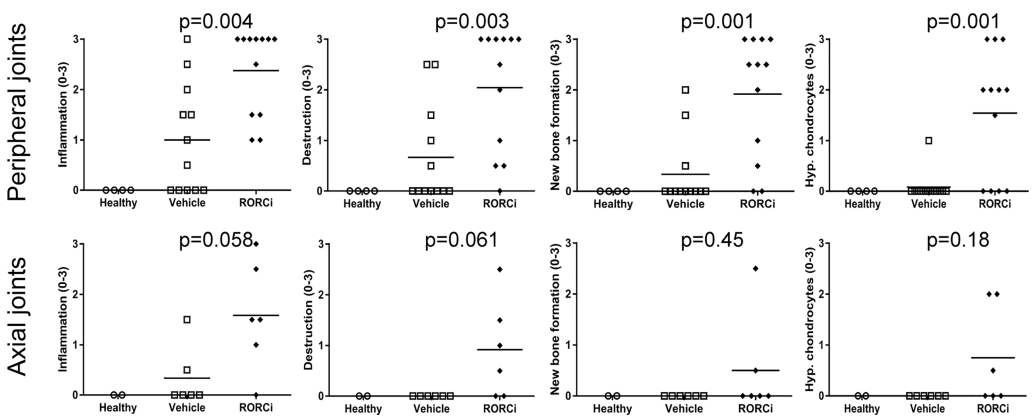

FIGURE 4 | Increased histological pathology in RORC inhibitor treated rats. Peripheral and axial joints were semi-quantitatively scored (0–3) for inflammation, destruction, new bone formation and hypertrophic chondrocytes.

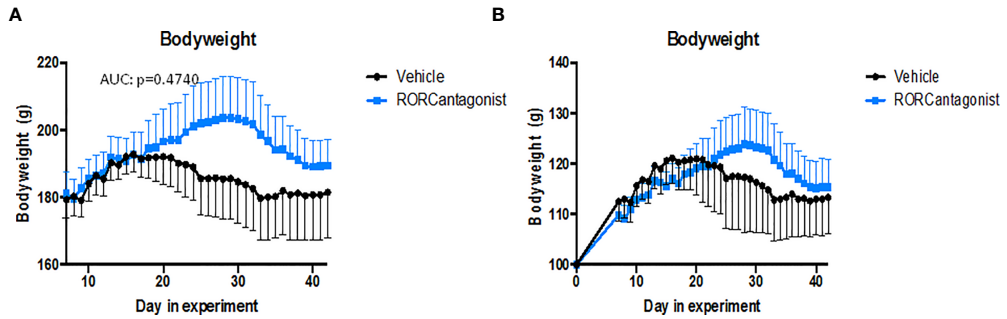

FIGURE 5 | Body weight follow up in treated rats. **(A)** experiment 1. **(B)** experiment 2.

Th17 response. The equilibrium between RORγt and FoxP3 expression level would equally control Treg and Th17 cells, depending on the cytokine environment. RORγt inhibition would twist the Th17/Treg cell ratio towards the Treg pathway (46). Despite the apparent beneficial effect for inducing immune tolerance, it seems that RORγt modulation would alter both the downstream pro- and anti-inflammatory pathways and induce undesirable effects (45). Affecting the regulatory function of these RORγt Tregs would predispose to inflammation (even when the expression of Th17 signature genes and cytokines is decreased). The question remains to what extend these findings are relevant for other models or for human disease. Based on our previous findings regarding IL-23R and IL-17A blockade in the HLA-B27 tg rats we conclude that it is uncertain whether RORC inhibition might be a good therapeutic option for all IL-17A driven diseases.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

All animal experiments were approved by the AMC Animal Care and Use Committee, in line with national and international regulations and guidelines.

AUTHOR CONTRIBUTIONS

MT and LD, study design, experimental procedures, analyzing data, writing manuscript. MM, analyzing data, writing manuscript. JW, experimental procedures. ML, study design, experimental procedures, analyzing data. MS, analyzing data, writing manuscript. GN and DB, study design, analyzing data, writing manuscript. All authors contributed to the article and approved the submitted version.

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The Clinical and MRI Effect of TNF-α Inhibitors in Spondyloarthritis Patients With Hip Involvement: A Real-World Observational Clinical Study

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Objectives: Hip involvement is an important cause of disability and poor prognosis in patients with spondyloarthritis (SpA). Tumor necrosis factor (TNF)-α inhibitor treatment has been demonstrated to be effective in SpA patients with hip arthritis; however, quantitative assessment using MRI in long-term follow-up needs further application and observation.

Methods: A total of 239 patients were involved in this study. Methotrexate and sulfasalazine were given as basic treatment. In total, 165 patients received TNF-α inhibitors plus basic treatment, and 74 received basic treatment only, as controls. Clinical symptoms were assessed at baseline and at weeks 12, 24, and 52. MRI performances of hip arthritis, including bone marrow edema (BME) and synovitis, were quantitatively assessed using the Hip Inflammation MRI Scoring System (HIMRISS).

Results: The clinical values of erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), Harris hip score, and Ankylosing Spondylitis Disease Activity Score (ASDAS)-ESR in both groups showed significant clinical remission at week 52 ($p < 0.001$). However, the change in disease activity levels at week 52 in the control group was significantly worse than in the TNF-α inhibitor group. At week 52, MRI showed a significant remission trend in the TNF-α inhibitor group versus baseline, and total HIMRISS scores were significantly decreased (26.49 ± 10.37 *vs.* 20.59 ± 9.41, $p < 0.001$); the control group only had slight improvement ($p < 0.05$).

Conclusions: TNF-α inhibitors could significantly improve clinical and MRI manifestations of hip involvement in patients with SpA. Quantitative MRI assessment combined with clinical assessment can be used to accurately evaluate the treatment effect of TNF-α in SpA patients with hip involvement to help guide targeted treatment.

Keywords: MRI, hip, spondyloarthritis, TNF-α, ASDAS-ESR

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INTRODUCTION

Hip involvement is an important cause of disability and poor prognosis in approximately 10%–50% of patients with spondyloarthritis (SpA) (1, 2), and 47%–90% of those patients with hip involvement have it bilaterally (3). Progression of hip involvement is reported to be associated with more severe spinal involvement (4, 5) and seriously affects joint function and life quality. In addition, nearly 8% of patients with hip involvement have intractable pain and disability. Even if they are treated with total hip arthroplasty, there is a high likelihood of revision surgery and high rates of complication (6).

The principal clinical manifestations of hip involvement in SpA include inflammation of the subchondral bone marrow edema (BME) and synovitis effusion (7). MRI has been widely used as a sensitive tool to detect hip arthritis. MRI is helpful for early diagnosis to enable treatment to suppress inflammation and avoid further structural damage (8, 9). The Hip Inflammation MRI Scoring System (HIMRISS) is a new quantitative assessment method that is based on several MRI slices from one patient. The HIMRISS is used to systematically assess synovitis and BME of the femoral head and acetabulum (10). Although the association between the HIMRISS and disease activity has been validated in SpA (11), the sensitivity and accuracy of the HIMRISS in assessing treatment response in SpA with hip involvement require further validation.

Previous SpA treatment strategies have focused on global symptom management rather than SpA-related hip lesions or other peripheral joint diseases (12, 13). Increasing evidence indicates that early detection and diagnosis of hip inflammation are beneficial to active treatment, which is important to improve hip function and general prognosis of SpA (14). Tumor necrosis factor (TNF)-α inhibitors are a prompt and robust treatment to improve the signs and symptoms of SpA as well as function and spinal mobility (15, 16). In recent clinical studies, TNF-α inhibitors, including etanercept, adalimumab, and infliximab, were shown to be effective for patients with SpA patients with hip involvement in clinical and imaging assessments (1). However, the detailed clinical effects of TNF-α inhibitors in the long-term treatment cycle need to be further clarified. Previous imaging assessments have been limited to radiographic assessment of Bath Ankylosing Spondylitis Radiology Index (BASRI)-hip or ultrasound (17, 18). More accurate imaging tools such as MRI are not sufficiently applied in assessment of the effect of acute and long-term follow-up treatment with TNF-α inhibitors.

Thus, the purpose of this prospective study was to quantitatively assess the details of clinical remission and MRI changes in hip involvement under treatment with TNF-α inhibitors. Hip inflammation was quantitatively assessed using HIMRISS based on MRI changes, as well as systematic clinical evaluation. We aimed to suggest an assessment standard for patients with SpA patients with hip involvement, including MRI and clinical standards, to help guide accurate and targeted treatment.

Abbreviations: SpA, spondyloarthritis; BME, bone marrow edema; HIMRISS, Hip Inflammation MRI Scoring System; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; ASDAS, Ankylosing Spondylitis Disease Activity Score; ASAS, Assessment of SpondyloArthritis international Society; STIR, short tau inversion recovery; DMARDs, disease-modifying anti-rheumatic drugs; MTX, methotrexate; SSZ, sulfasalazine; HLA-B27, human leukocyte antigen B27; BASRI-h, Bath Ankylosing Spondylitis Radiology Index-hip score.

MATERIALS AND METHODS

Study Population

This clinical trial was registered in the Chinese Clinical Trial Registry on November 28, 2011 (ID: ChiCTR-ONRC-11001846, http://www.chictr.org.cn/showproj.aspx?proj=7701). The study was based on data from Xijing Hospital in China. All patients with a clinical diagnosis of SpA were enrolled, and long-term treatment and monitoring were conducted by rheumatologists in Xijing Hospital. Eligible patients were 18–65 years of age and fulfilled the recently published Assessment of SpondyloArthritis international Society (ASAS) classification criteria (19) or the Modified New York criteria for SpA (20).

The inclusion criteria for hip involvement were subject with i) spontaneous groin, thigh, and hip pain with or without history of trauma; ii) limited internal and external rotation of the symptomatic hip/hips after initial clinical assessment; and iii) both acute and chronic inflammatory changes on MRI.

Patients were excluded from this study if they had i) hip surgery or hip trauma in the previous year; ii) pregnancy; iii) any condition that would limit lower extremity function and mobility, such as history of stroke, infection, lower extremity joint replacement, or amputation; iv) abnormal laboratory test at 4 weeks before treatment (routine blood test: hemoglobin <90.0 g/L, white blood cell count $<3.0 \times 10^9$/L, and neutrophils $<1.5 \times 10^9$/L; biochemical test: alanine aminotransferase (ALT) or aspartate aminotransferase (AST) or total bilirubin >normal upper limit, and positive for HIV or syphilis); v) MRI contraindications (e.g., implanted pacemaker or claustrophobia); or vi) if the acquired MRI images were suboptimal in quality.

In order to enhance treatment of SpA patients with hip involvement and reduce disability rate, all enrolled participants would receive basic treatment with methotrexate (10 mg every week) and sulfasalazine (SSZ; 2 g/day, given orally) from baseline to 52 weeks. For all eligible participants, patients who were disinclined to have TNF-α inhibitor or with TNF-α inhibitor contraindications such as the patients with a hepatitis B virus and mycobacterium tuberculosis infection were allocated to the control group and would receive basic treatment only. Other patients were allocated to the TNF-α group and received one of three kinds of TNF-α inhibitor plus basic treatment (etanercept 50 mg given once weekly subcutaneously (s.c.); adalimumab treatment 40 mg s.c. every other week; infliximab 3 mg/kg i.v. at baseline, week 2 and week 6, and then every 8 weeks). The cost of treatment was self-funded, and therapeutic options were determined on the consent of the doctors and patients. The study was approved by the ethics committee of Xijing Hospital, and all patients provided written informed consent before enrollment in this study.

Erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and human leukocyte antigen B27 (HLA-B27) were measured with standard laboratory techniques. Serum CRP and ESR were measured at baseline and at 12, 24, and 52 weeks. At the same time point, evaluation of disease activity levels (Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), Ankylosing Spondylitis Disease Activity Score (ASDAS)-CRP, and ASDAS-ESR) and hip function level [Harris hip score (HHS)] were conducted by experienced rheumatologists. At the same time point, the ASAS remission rates (including ASAS20, ASAS 40, ASAS50, and ASAS partial) were also calculated for the TNF-α group.

Quantitative MRI Assessment of Changes

The HIMRISS was used to assess the changes on MRI between baseline and week 52. MRI was performed with a coronal short tau inversion recovery (STIR) sequence with slice thickness of 4 mm and field of view 400 × 400 mm. The baseline and follow-up MRI examinations of both hips were recommended to be performed every 6 months during the 52-week follow-up. And the MRI scans at baseline and at week 52 were calculated by HIMRISS. The HIMRISS protocol was used to score the inflammatory changes on MRI images of the hip joints including BME of the femoral head, BME of the acetabulum, and synovitis (10, 11). The HIMRISS procedure was performed independently by two readers (YL and MZ) who had been well trained at a previous study (11). The final HIMRISS scores were mean scores of both readers. Statistical analysis was conducted by an independent technologist (YZ). The standard HIMRISS scores were calculated based on 15 MRI image slides; therefore, to avoid bias, patients with MRI images of the hip structure who had fewer than five slides were considered as missing samples.

Statistical Analysis

The Kaplan–Meier test was used to assess the differences in remission of clinical symptoms (ASAS20, ASAS40, ASAS50, and ASAS partial) and MRI improvement between different follow-up time points. The baseline status was taken into account, and non-parametric analysis of covariance was used. The statistical analyses were performed using IBM SPSS 22.0 (IBM Corp., Armonk, NY, USA). p-Values <0.05 were considered to be statistically significant.

RESULTS

Demographic and Clinical Characteristics

Between January 1, 2014, and December 30, 2018, we enrolled 239 patients with SpA and coxitis, and treatment follow-up was completed in these patients. In total, 165 patients (69.04%) received TNF-α treatment, and 74 controls (30.96%) received basic treatment only (**Table 1**). In the TNF-α inhibitor group, 103 patients received etanercept treatment, 51 received adalimumab, and 11 patients received infliximab treatment. Patients with SpA in both the TNF-α inhibitor group and control group underwent evaluation of clinical symptoms and MRI examination during the 52-week observation period. As shown in **Table 1**, the differences in demographic and clinical characteristics including ESR, CRP, Harris, BASDAI, morning stiffness time, and ASDAS-CRP between the two groups were not significant at baseline, except for ASAS-ESR ($p < 0.01$) and disease duration ($p < 0.001$). Patients in the TNF-α inhibitor group had shorter disease duration and worse disease activity than the control group. All patients had serious disease activity with high BASDAI scores (5.76 ± 1.20 and 5.53 ± 1.29) and ASDAS score (2.78 ± 0.62 and 2.53 ± 0.57), as well as elevated CRP and ESR values.

TABLE 1 | Clinical characteristics including disease activity indexes and hip function in both groups at baseline.

	TNF-α group	Control group	p-Value
Number	165	74	
Age	28.67 ± 10.21	27.3 ± 7.11	0.362
Sex	117:48 = 71%:39%	43:31 = 58%:42%	0.067
B27	93%	87%	0.163
Disease duration (m)	25.19 ± 34.52	45.41 ± 40.48	0.000
ESR (mm)	26.91 ± 14.90	25.04 ± 14.34	0.367
CRP (mg/L)	1.42 ± 1.28	1.27 ± 1.23	0.364
Harris	67.65 ± 9.26	66.27 ± 10.85	0.313
BASDAI	5.76 ± 1.20	5.53 ± 1.29	0.199
Morning stiffness (h)	0.43 ± 0.18	0.39 ± 0.42	0.276
ASDAS-ESR	2.76 ± 0.60	2.90 ± 0.67	0.124
ASDAS-CRP	2.78 ± 0.62	2.53 ± 0.57	0.004

ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; ASAS, Assessment of SpondyloArthritis international Society.

Quantitative MRI Assessment

MRI changes in hip inflammation among patients with SpA were assessed using the HIMRISS, including BME and synovitis. Compared with those at baseline, patients in the TNF-α inhibitor group had significant improvement on MRI of both BME and synovitis at week 52 (all $p < 0.001$). However, the difference on MRI between week 52 and baseline in the control group was smaller (BME of the femoral head, $p < 0.05$; total BME, $p < 0.05$; synovitis, $p < 0.05$; total mean/max HIMRISS, $p < 0.05$); BME scores of the acetabulum were similar ($p > 0.01$) (**Table 2**).

Intergroup analysis showed little difference between the total HIMRISS scores of the TNF group and control group at baseline ($p > 0.05$) and at week 52 ($p > 0.05$). However, the treatment group with higher total HIMRISS scores at baseline demonstrated significant improvement at week 52 and lower scores than did the control group (TNF-α *vs.* control groups at week 52: 20.59 ± 9.41 *vs.* 22.33 ± 7.0). Additionally, acetabular BME scores indicated that patients in the TNF-α group had significantly more serious disease activity at baseline ($p = 0.006$) but had significant improvement at week 52 ($p < 0.001$) (**Table 2**).

Clinical Symptoms and Hip Function Assessment

Both the TNF-α inhibitor group and control group achieved significant amelioration of all clinical symptoms and hip function at week 52, but the improvement degrees of TNF

TABLE 2 | HIMRISS values from baseline to 52 weeks in the biological group and non-biological group.

	Group	Baseline	Treatment 52 weeks	Changed levels	Baseline *vs.* week 52 p-value
BME of femoral head^{mean}	TNF-α group	10.53 ± 6.78	7.74 ± 5.77	2.86 ± 0.38	0.000
(0–65)	Control group	9.74 ± 5.77	8.73 ± 5.06	0.74 ± 0.40	0.018
	TNF *vs.* control p-value	0.124	0.315	0.001	
BME of acetabular^{mean}	TNF-α group	7.06 ± 3.68	5.51 ± 3.81	1.60 ± 0.23	0.000
(0–35)	Control group	6.27 ± 2.82	5.89 ± 2.45	0.33 ± 0.26	0.164
	TNF *vs.* control p-value	0.006	0.000	0.028	
Total BME^{mean}	TNF-α group	17.59 ± 9.36	13.26 ± 8.75	4.46 ± 0.52	0.000
(0–100)	Control group	16.05 ± 7.11	14.61 ± 2.44	1.06 ± 0.55	0.019
	TNF *vs.* control p-value	0.047	0.037	0.005	
Synovitis effusion score^{mean}	TNF-α group	8.90 ± 3.33	7.34 ± 2.97	1.53 ± 0.23	0.000
(0–30)	Control group	8.44 ± 2.19	7.71 ± 2.24	0.48 ± 0.23	0.011
	TNF *vs.* control p-value	0.002	0.088	0.000	
Total HIMRISS^{ave}	TNF-α group	26.49 ± 10.37	20.59 ± 9.41	5.99 ± 0.58	0.000
(0–130)	Control group	24.49 ± 8.07	22.33 ± 7.07	1.54 ± 0.66	0.039
	TNF *vs.* control p-value	0.073	0.079	0.011	
Total HIMRISS^{max}	TNF-α group	29.95 ± 11.70	23.03 ± 10.31	7.07 ± 0.66	0.000
(0–130)	Control group	27.31 ± 9.50	24.64 ± 8.38	1.91 ± 0.73	0.015
	TNF *vs.* control p-value	0.118	0.126	0.007	

ave, the average score of two hips; max, the max score of two hips; changed levels, score at baseline minus score at week 52; HIMRISS, Hip Inflammation MRI Scoring System; BME, bone marrow edema.

group were significantly better than those of the control group (**Table 3**). Notably in TNF-α inhibitor group, BASDAI scores decreased from 5.76 ± 1.20 to 2.16 ± 1.90 at week 52 ($p < 0.001$); ASDAS-CRP/ASDAS-ESR decreased from 2.78 ± 0.62/2.76 ± 0.60 to 1.06 ± 0.46/1.42 ± 0.60 at week 52 ($p < 0.001$); CRP and ESR levels also improved significantly compared with baseline levels ($p < 0.001$ and $p < 0.001$). In addition, Harris score indicated significant hip function improvement to generally normal by week 52 versus baseline in the TNF-α inhibitor group (67.65 ± 9.26 *vs.* 92.26 ± 7.06, $p < 0.001$).

Even the control group showed a significant clinical improvement at week 52 versus baseline ($p < 0.001$); the mean BASDAI score, ASDAS-CRP, ASDAS-ESR, and Harris scores of the TNF-α group were significantly better than those of the control group at week 52 (all p-values <0.001) (**Table 3**). In particular, the degree of ASDAS-CRP indicated a real-world clinical characteristic. Patients in the TNF-α group had more serious disease activity at baseline but obviously better remission at week 52 than had controls.

The ASAS remission rates of ASAS20, ASAS40, and ASAS50 and the ASAS partial remission rate were compared between the TNF-α group and control group at week 52. The TNF-α group had higher and better ASAS remission levels than the control group (**Figure 1**). Moreover, the distribution of disease activity including BASDAI, ASDAS-CRP, and ASDAS-ESR between the two groups at week 52 was significantly different. Patients in the TNF-α inhibitor treatment group were able to achieve inactive disease status (TNF-α *vs.* controls: BASDAI, 96.93% *vs.* 74.33%; ASDAS-CRP, 80.98% *vs.* 58.67%; ASDAS-ESR, 51.53% *vs.* 22.67%; $p < 0.001$). However, a considerable number of patients in the control group were still classified as having

TABLE 3 | Clinical outcomes and hip function from baseline to 52 weeks between the TNF-α inhibitor group and control group.

	Groups	Baseline	Treatment 52 weeks	p-Value
ESR (mm)	TNF-α group	26.91 ± 14.90	11.01 ± 12.58	0.000
	Control group	25.04 ± 14.34	17.07 ± 12.23	0.000
	p-Value	0.367	0.002	
CRP (mg/L)	TNF-α group	1.42 ± 1.28	0.73 ± 1.17	0.000
	Control group	1.27 ± 1.23	0.88 ± 0.91	0.047
	p-Value	0.364	0.371	
Harris	TNF-α group	67.65 ± 9.26	92.26 ± 7.06	0.000
	Control group	66.27 ± 10.85	82.95 ± 7.93	0.000
	p-Value	0.313	0.000	
BASDAI	TNF-α group	5.76 ± 1.20	2.16 ± 1.90	0.000
	Control group	5.53 ± 1.29	2.81 ± 1.42	0.000
	p-Value	0.199	0.000	
ASDAS-CRP	TNF-α group	2.78 ± 0.62	1.06 ± 0.46	0.000
	Control group	2.53 ± 0.57	1.40 ± 0.63	0.000
	p-Value	0.004	0.000	
ASDAS-ESR	TNF-α group	2.76 ± 0.60	1.42 ± 0.60	0.000
	Control group	2.90 ± 0.67	1.88 ± 0.62	0.000
	p-Value	0.124	0.000	

ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; ASDAS, Ankylosing Spondylitis Disease Activity Score.

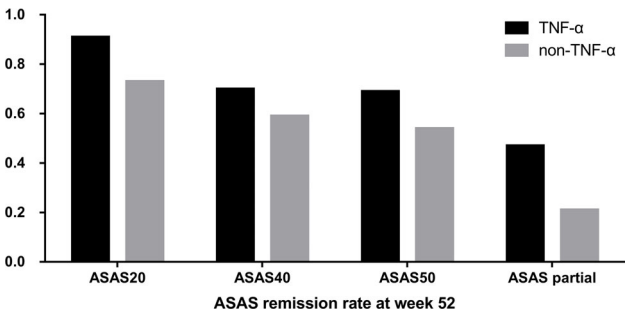

FIGURE 1 | Distribution of Assessment of SpondyloArthritis international Society (ASAS) remission rates between the group that received tumor necrosis factor (TNF)-α inhibitors and the control group at week 52.

active disease (TNF-α *vs.* controls: BASDAI, 3.07% *vs.* 22.67%; $p < 0.001$), low disease activity (TNF-α *vs.* controls: ASDAS-CRP, 15.34% *vs.* 24.00%; ASDAS-ESR, 35.58% *vs.* 42.67%; $p < 0.001$), and moderate disease activity (TNF-α *vs.* controls: ASDAS-CRP, 3.07% *vs.* 17.33%; ASDAS-ESR, 11.66% *vs.* 32.00%; $p < 0.001$) (**Figure 2**).

We also performed subgroup analysis of all patients in the TNF-α group to compare the treatment difference between different TNF-α inhibitors. First, for all patients receiving treatment with TNF-α inhibitors, all continuous parameters related to disease activity (i.e., CRP, ESR, BASDAI, and ASDAS) and hip joint function (i.e., Harris) were significantly ameliorated from week 12 (all $p < 0.001$) compared with baseline, and the significant remission levels persisted until week 52 (all $p < 0.001$) (**Figure 3**). ASAS remission rates, including those of ASAS20, ASAS40, and ASAS50, and ASAS partial remission rates were compared between different TNF-α inhibitors and follow-up times. The results indicated that the differences in ASAS remission rates between different TNF-α inhibitor groups during the 52 weeks were not significant ($p > 0.01$) (**Figure 4**). Additionally, the HIMRISS was also compared between different TNF-α inhibitor groups (**Supplementary Table S1**). Both TNF-α inhibitor groups had achieved significant imaging improvement at week 52, and the changed HIMRISS levels were similar between different TNF-α inhibitor groups. The treatment responses were similar, which may be consistent with the clinical presentations.

Further, correlation analysis between HIMRISS and clinical outcome was also performed for both groups (**Supplementary Table S2**). In the TNF-α group, HIMRISS was significantly correlated with ASDAS-ESR, ASDAS-CRP, BASDAI, ESR, and CRP levels (all $p < 0.05$) in the TNF-α group at week 52, but the correlations were not significant at baseline. In addition, changed levels of HIMRISS between baseline and week 52 were significantly correlated with ASDAS-ESR changes in the TNF-α group. In the control group, HIMRISS was not significantly correlated with clinical outcomes at baseline and week 52, as well as changed levels (**Supplementary Table S2**).

DISCUSSION

This study demonstrated that treatment with TNF-α inhibitors could achieve significant clinical remission in patients and can manifest changes on MRI for hip arthritis in patients with SpA. In the TNF-α inhibitor group, disease activity indexes including BASDAI, ASDAS, ASAS remission rate, and hip function demonstrated superior improvement as compared with the control group. Additionally, the HIMRISS method could detect obviously better improvement in hip inflammation, based on overall MRI slices, under TNF-α inhibitor treatment versus control treatment. TNF-α inhibitor therapy was confirmed to be a better treatment option for patients with SpA and hip involvement.

Previous studies of therapy in SpA have mainly focused on axial spine radiology, and, to some extent, peripheral arthritis or enthesitis (5, 21). Hip involvement is difficult to cure, and it is considered an important cause of disability and poor prognosis in SpA, especially in ankylosing spondylitis (AS) (17, 22). There has been greater attention recently regarding hip arthritis in SpA, and various clinical studies on TNF-α inhibitor treatment and classical disease-modifying anti-rheumatic drug (DMARD) treatment have been conducted to assess the clinical and/or radiographic progress of hip joint and function (23–25). TNF-α inhibitor treatment has been demonstrated to be effective in patients with SpA and hip involvement. However, the specific effect differences between TNF-α inhibitors and other agents have rarely been evaluated in long-term follow-up studies.

Among therapeutic studies of SpA patients with hip involvement, the BASRI-h method is often adopted to assess radiological changes of hip lesions (24). However, most hip involvement in AS is related to early coxitis and is characterized by inflammation of the subchondral BME and synovitis (7, 26). The BASRI-h method is performed based on structural damage, which may not sensitively reflect the treatment effect in patients during early and acute inflammation stages in short-time follow-up. Comparatively, MRI is considered to be a more sensitive assessment tool for

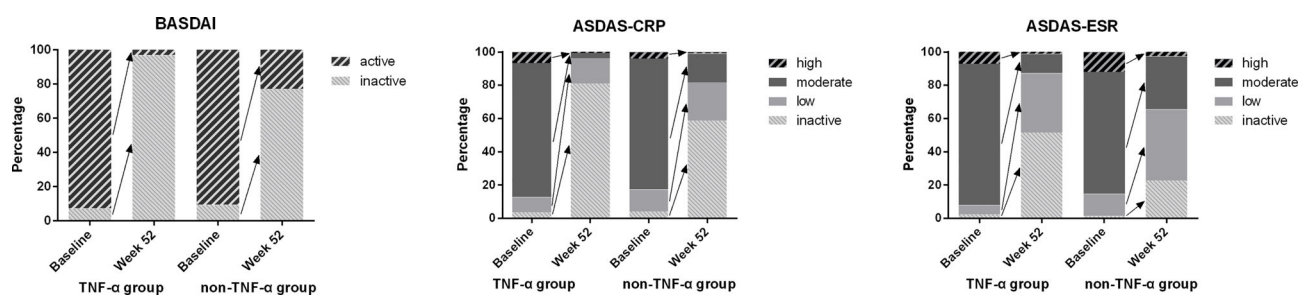

FIGURE 2 | Distribution of disease activity between baseline and week 52 in both groups. Notes. Inactive disease: ASDAS-CRP ≤ 1.3, ASDAS-ESR ≤ 1.3, or BASDAI ≤ 4. Low disease activity: 1.3 < ASDAS-CRP/ASDAS-ESR ≤ 2.1. Moderate disease activity: 2.1 < ASDAS-CRP/ASDAS-ESR ≤ 3.5. High disease activity: ASDAS-CRP/ASDAS-ESR > 3.5. Active disease: BASDAI > 4. ASDAS, Ankylosing Spondylitis Disease Activity Score; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index.

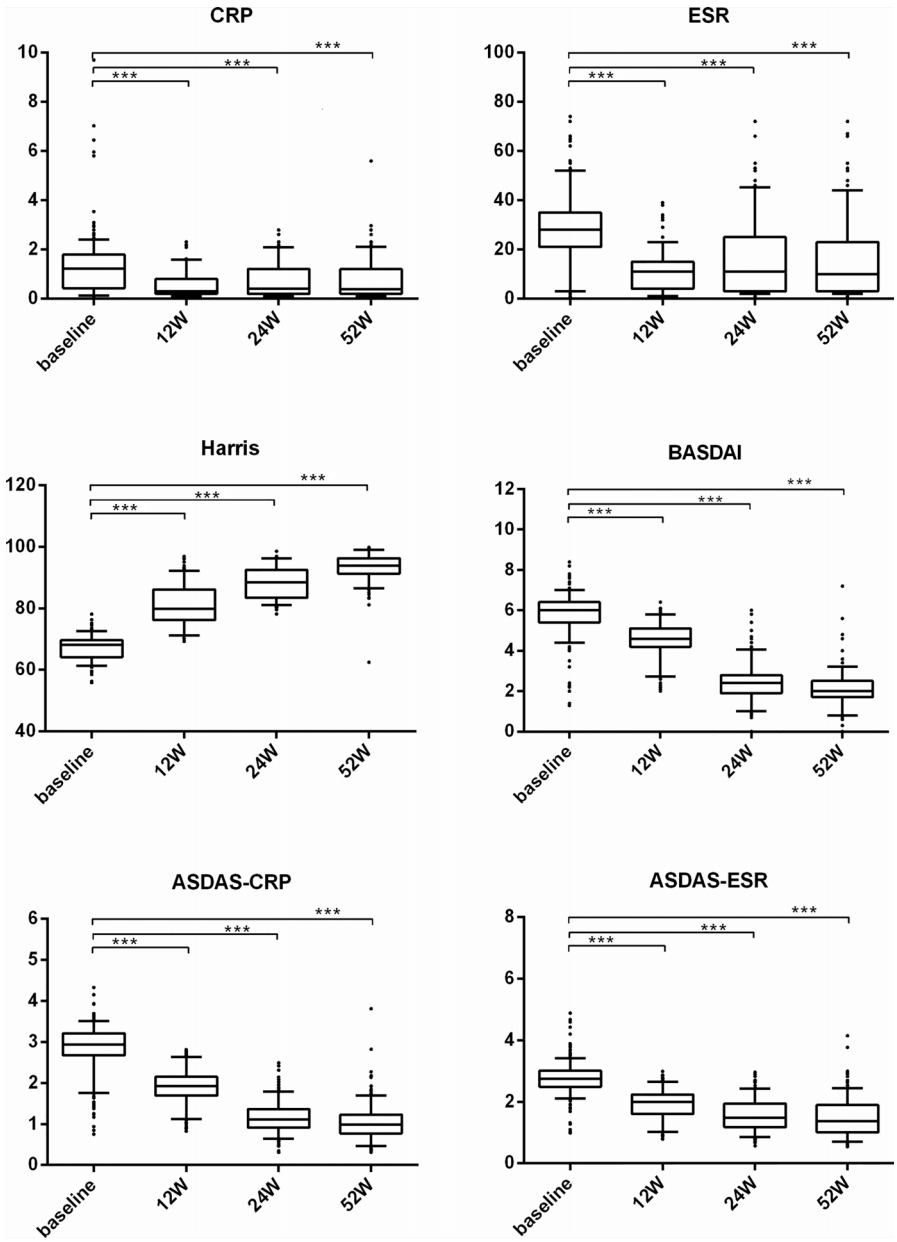

FIGURE 3 | The clinical performance of TNF-α inhibitor group from baseline to week 52. For each box-and-whisker plot, the whisker represents the range, the box represents the 5th–95th percentile, the solid lines within the box represent the mean values of clinical indexes, ***p < 0.001.

hip inflammation and is especially helpful in early diagnosis (8, 23, 27).

Recent studies have shown that synovial enhancement with hyperintense signals on contrast-enhanced T1-weighted images is the most frequent imaging finding in early-stage hip involvement in AS (14). Joint effusion of the femoral neck and subchondral BME are frequent findings with hyperintense signals on T2-weighted images or STIR images (8, 27). However, lesion frequency analysis or description of characteristics cannot quantitatively reflect MRI changes under treatment. The HIMRISS can be used to systematically quantify inflammatory changes including BME of the femoral head, acetabular BME, and synovitis effusion of the hip based on overall MRI slices (10). The HIMRISS method has been previously applied in patients with SpA diagnosed with hip arthritis, and there is a significant correlation between MRI manifestations and Harris score or disease activity (11).

In past clinical studies, clinical manifestations assessed in patients with SpA and hip involvement mostly refer to global outcome indexes, including disease activity scores and

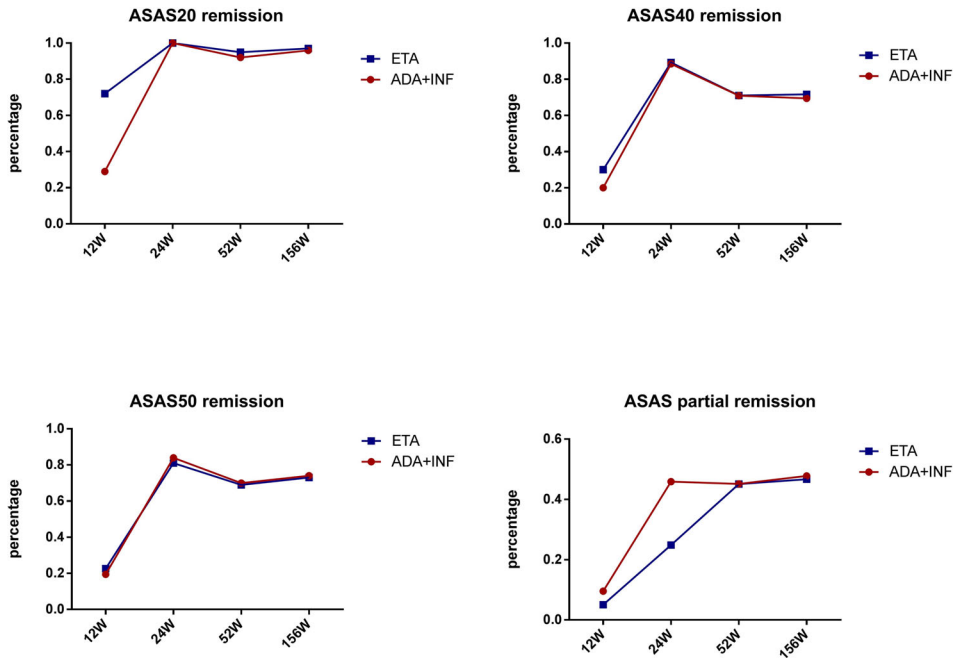

FIGURE 4 | ASAS remission rates between groups receiving different TNF-α inhibitors. ETA, etanercept; ADA, adalimumab; INF, infliximab; ASAS, Assessment of SpondyloArthritis international Society.

inflammatory markers. Despite the fact that the association between hip impairment and axial disease in inflammatory back pain is still controversial (22), further evidence points to the fact that hip disease more frequently occurs in patients with SpA and severe axial disease (28). However, the treatment efficacy and changes in hip involvement during the early stage and long-term follow-up are still not fully understood. Thus, more detailed assessments of clinical and MRI manifestations are needed.

In this study, the clinical efficacy of TNF-α inhibitors was compared with that of control agents in a 1-year follow-up period. TNF-α inhibitors were demonstrated to be significantly more effective in improving disease activity and inflammation than usual DMARD treatment. All the clinical indexes of the TNF-α group including ESR, CRP, BASDAI, and ASDAS-CRP/ASDAS-ESR were significantly ameliorated at different follow-up times compared with baseline; this result is consistent with previous studies. Harris score was also significantly increased, which reflected outstanding improvement of hip function.

In this study, the HIMRISS method was used to quantify the acute and chronic inflammatory changes of hip arthritis in SpA under treatment with TNF-α inhibitors or control treatment. The results of the HIMRISS showed significantly better improvement on MRI in the TNF-α group than the control group at week 52. All HIMRISS parameters of the TNF-α group including BME of the femoral head, BME of the acetabulum, synovitis effusion, and total HIMRISS scores showed significant improvement, with p-values <0.001. These results indicated that, to some extent, TNF-α inhibitors could simultaneously achieve significant long-term remission on MRI and clinical remission versus control treatment, which showed no notable MRI remission. Thus, TNF-α inhibitors demonstrated outstanding advantages for patients with SpA and hip involvement.

We performed additional subgroup analyses to further compare the clinical effect among different TNF-α inhibitors. A previous study indicated that etanercept and adalimumab treatment led to similar ASDAS-CRP improvement in patients with AS (16). In this study, the ASAS remission rates and HIMRISS were simultaneously compared between etanercept/infliximab and adalimumab at follow-up time points. Results of the Kaplan–Meier test indicated that the difference in remission rates, including those of ASAS20, ASAS40, and ASAS50, and the ASAS partial remission rate were not significant among the different TNF-α inhibitors. At the same time, HIMRISS also had a similar improvement at week 52 between different TNF-α inhibitors with all p-values >0.5. This result indicated that all TNF-α inhibitors had a similar positive effect in patients with SpA complicated with hip involvement.

The follow-up cycle of hip joint function and clinical characteristics varies from 12 weeks to 24 months. In this study, we observed the treatment response between 12 and 52 weeks. Lian et al. and Huang et al. (23) demonstrated that TNF-α inhibitors could maintain stable hip function according to the Harris and clinical remission of hip arthritis in patients with SpA for 6 months. In this study, we simultaneously compared the effectiveness of TNF-α inhibitors and control therapy for the clinical outcome of hip arthritis in SpA from baseline to 52 weeks. Our results further confirmed that clinical remission levels at week 52 in the TNF-α inhibitor group were significantly better than those of the SSZ group in all clinical

parameters including ESR, CRP, Harris, BASDAI, and ASDAS. TNF-α inhibitor treatment yielded rapid clinical remission within 12 weeks and hip function improvement, and this improvement tended to remain through week 52.

The correlation analysis between HIMRISS and systematic clinical symptoms only indicates significant correlations in TNF-α inhibitor group at week 52, but the correlations were not significant at other time points and not significant in the control group. This result indicated that clinical remission and imaging remission may not be achieved at the same time, and treatment effect needs to be evaluated by multi-aspects.

Based on the fact that the cost of TNF-α inhibitors is relatively high in comparison with that of classical DMARDs, the present results indicate a real-world characteristic at baseline that patients in the TNF-α group had more serious disease activity and shorter disease duration than those in the control group, which was consistent with previous studies (29). Patients with SpA who had active hip arthritis are recommended to receive TNF-α inhibitor therapy for quick pain reduction and symptom remission, based on the global effectiveness of TNF-α inhibitors (30). Although disease duration assessed by months at baseline is significantly longer in the control group than the TNF-α group, the difference of average disease duration between the two groups was less than 2 years. In addition, the systematic clinical indexes of ESR, CRP, B27 Harris, BASDAI, and ASDAS-CRP at baseline were not significantly different between two groups. To some extent, the disease activity is a little worse in the TNF group instead. The results showed that as a chronic disease, although the average duration of disease difference in SpA between the two groups was less than 2 years, it should not have a great impact on the treatment response in this study. Similarly, the baseline results of MRI analysis did not fulfill the criteria of no difference between the two groups, particularly regarding the parameters of acetabular BME score, total BME score, and effusion synovitis. The present real-world data revealed that both disease activity and MRI features in patients under treatment with TNF-α inhibitors are more serious. TNF-α inhibitor therapy is suggested in these patients to quickly improve clinical outcomes, relieve pain, and reduce adverse reactions. As a consequence of these real-world characteristics, the present results must be interpreted with caution.

Some limitations of this study should be acknowledged. First, the 1-year treatment and observation period were short; longer follow-up is needed. Next, this was a single-center study in real world, and population bias may influence the applications regarding treatment efficiency. Moreover, although the HIMRISS method was used based on accurate MRI slices, it is not commonly used; additional application and confirmation of this method are required in the future.

CONCLUSION

The results of our quantitative assessment demonstrated that TNF-α inhibitors could significantly and simultaneously improve clinical outcomes and the treatment response on MRI for hip arthritis in patients with SpA. This improvement was achieved quickly, within a 12-week treatment period, and was maintained in long-term follow-up. Our findings support a superior curative effect of TNF-α inhibitors over non-TNF-α inhibitor agents.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committee of Xijing Hospital of China (instruction number: 20110303-7). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KZ, YZ, and PZ contributed to the completion of the first draft and final version of the manuscript. PZ, KZ, and ZZ contributed to the initial design of the work. KZ, JD, ZZ, JJ, and PZ contributed to the clinical validation of the follow-up data. YL and MZ contributed to the interpretation of the MRI data. YZ, QH, WW, YW, and BZ contributed to the data acquisition and analysis. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.740980/full#supplementary-material

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SYSTEMATIC REVIEW
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Association Between Infections and Risk of Ankylosing Spondylitis: A Systematic Review and Meta-Analysis

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Background: Previous literature on the association between infections and the risk of developing ankylosing spondylitis (AS) presented controversial results. This meta-analysis aimed to quantitatively investigate the effect of infections on the risk of AS.

Methods: We searched the PubMed, Embase, and Web of Science databases until March 26, 2021 for analytical epidemiological studies on the association between infections and the risk of AS. Fixed or random effect models were used to calculate total risk estimates based on study heterogeneity. Subgroup analysis, and sensitivity analysis were also performed. Publication bias was estimated using funnel plots and Begg's test.

Results: Six case-control articles (n=1,296,239) and seven cohort articles (n=7,618,524) were incorporated into our meta-analysis. The pooled odds ratio (OR) from these case-control studies showed that infections were associated with an increased risk of AS (OR=1.46, 95% confidence interval [CI], 1.23–1.73), and the pooled relative risk (RR) from the cohort studies showed the same findings (RR=1.35, 95% CI, 1.12–1.63). Subgroup analysis showed that infections in participants with unadjusted comorbidities (OR=1.66, 95% CI, 1.35–2.03), other types of infection (OR=1.40, 95% CI, 1.15–1.70), and infection of the immune system (OR=1.46, 95% CI, 1.42–1.49) were associated with the risk of AS in case-control studies. In cohort studies, infections with adjusted comorbidities (RR=1.39, 95% CI, 1.15–1.68), viral infection (RR=1.43, 95% CI, 1.22–1.66), other types of infection (RR=1.44, 95% CI, 1.12–1.86), and other sites of infection (RR=1.36, 95% CI, 1.11–1.67) were associated with an increased risk of AS.

Conclusions: The findings of this meta-analysis confirm that infections significantly increase the risks of AS. This is helpful in providing an essential basis for the prevention of AS *via* the avoidance of infections.

Keywords: ankylosing spondylitis, infections, analytical epidemiology, systematic review, meta-analysis

INTRODUCTION

Ankylosing spondylitis (AS), a complex autoimmune inflammatory rheumatic disease, has long been considered the archetype of spondyloarthritis (SpA). Common symptoms of AS include arthritic symptoms (such as inflammatory back pain, muscle spasms, and sacroiliac arthritis), potential extra-articular symptoms (such as uveitis, psoriasis, and inflammatory bowel syndrome), and the involvement of the heart, bone, lung, kidneys, and skin (1, 2). The worldwide prevalence of AS ranges between 0.07% and 0.32% (3). In addition, clinical symptoms of patients with AS usually appear between the ages of 26 and 45 years. Men also are more likely to suffer from AS than women, the prevalence being two to three times higher in men than in women (4–6).

The pathogenesis of AS is complex and multifactorial. Early studies have confirmed that AS is strongly associated with the inheritance of HLA allele B27, which might misfold in the endoplasmic reticulum (ER), leading to the upregulation of interleukin (IL)-23 in dendritic cells (7–9). It may also result in the presentation of intracellular peptides to T cells, which may trigger cross reactions, leading to tissue inflammation (10, 11). Several recent studies have emphasized the critical role of intestinal flora dysregulation in the development and progression of AS, and have suggested that 60% of AS patients are associated with subclinical intestinal inflammation (12–14). This might be related to the imbalance of IL-17 or IL-23 cytokines caused by the activation a Th17-mediated immune response by intestinal dysbiosis (15). In contrast, the role of environmental factors in the etiology of AS is far from clear. One of the most popular theories presume that the onset of AS in susceptible individuals may be caused by infections (16), and that infections have the potential to modulate and attenuate immune responses.

The underlying pathogenic mechanisms for linking infections and AS involve changes in target cells and immune cells, and antigenic cross-reactions between microbial and host determinants (17). Infections might cause the quantitative reduction in specific T cells and the host defense defect against the infections that allows microbial antigens to reach the joint (18). The association between the infections and AS may be *via* IL-17 or C reactive protein levels that can induce inflammatory response (10, 19). In addition, certain microbial infections may reduce CD4+ T cells, and protein fragments released by dying CD4 lymphocytes may induce autoreactive CD8 lymphocytes (20). There is evidence of significantly elevated levels of IL-6 and TNF-a in AS patients, which might be caused by infections (21).

Numerous studies have investigated AS-related infections, including bacterial (10, 17, 18, 22), viral (17, 19, 20, 23), fungal (11), and those by microorganisms with sizes between those of bacteria and viruses (18, 24, 25). The infected sites include the respiratory (18, 24–26), immune (20, 23, 26, 27), digestive (10, 22, 26, 28), and genitourinary systems (19). However, there is no consensus on the association between infections and the risk of AS. To our knowledge, no systematic review and meta-analysis to date has investigated the effect of infections on the risk of AS. Therefore, in order to obtain a more convincing conclusion, this study aimed to review all the relevant studies and summarise the findings, in order to investigate the association between infections and AS.

MATERIALS AND METHODS

The current study was developed according to the guidelines for the Meta-Analysis of Observational Studies in Epidemiology (MOOSE) (29) and Preferred Reporting Items for Systematic Review and Meta-Analyses (PRISMA, **Supplementary Table 1**) (30). The protocol is presented in **Supplementary Data**.

Search Strategy

We systematically searched the PubMed, Embase, and Web of Science electronic databases to identify such literature published up until March 26, 2021, using terms related to infection and AS. The search strategy was developed and implemented under the guidance of experts on library services from Shandong First Medical University. The main search strategy involved the following: (spondylitis, ankylosing OR spondyloarthritis ankylopoietica OR ankylosing spondylarthritis OR ankylosing spondylarthritides OR spondylarthritides, ankylosing) AND (infections OR enteritis OR salmonella OR pneumonia OR klebsiella pneumoniae OR urogenital infections OR paradentitis OR tonsillitis OR infection of the upper respiratory tract OR appendicitis OR gastritis OR helicobactor pylori OR virus) AND (case-control study OR retrospective study OR cohort study OR prospective study OR longitudinal study OR follow-up study). The complete search strategy of the three databases is listed in **Supplementary Table 2**. Moreover, only English- or Chinese-language literature was retrieved from the databases as the investigators were proficient in both these languages. The lists of references from all of the included studies were manually checked to identify possible additional studies.

Selection Criteria

Studies were included according to the following criteria: (1) the study design was a cohort or case-control study; (2) the studies defined infections using self-reporting, clinical diagnosis, or basic medical experiment, and focused on infections that developed before AS did; (3) the outcome of interest was AS; and (4) the studies provided the effect size (relative risk [RR], hazard ratio [HR], or odds ratio [OR] with 95% confidence interval [CI]) or raw data that could be used to calculate RR, HR, or OR. The exclusion criteria were as follows: (1) non-human-based studies; (2) studies that were poster presentations, reviews, conference summaries, or dissertations; and (3) the scores of quality evaluation according to the Newcastle-Ottawa Scale (NOS) were <4 (31). In the situation of multiple eligible studies from the same population, only the study with the largest number of individuals was included. Two authors (X.Z. and A.Z.) independently screened titles and abstracts initially and then evaluated full-text articles to ensure the included studies met the eligible inclusion criteria. Any disagreement between them was settled by another author (G.D.).

Data Extraction and Quality Assessment

According to the study design, the included studies were divided into two extraction forms of case-control studies and cohort studies. The following data were extracted from the eligible case-control studies using a customized form: the first name of the first author, year of publication, study location, types of infection, definition of infection, definition of AS, age, sex, sample size, adjustment for potential confounding factors, and estimates of association. The follow-up duration in cohort studies was also included. The Cochrane Non-randomized Studies Methods Working Group recommended the use of the NOS to assess the quality of observational studies (range: 0–9 stars) (32). According to the score stars of the NOS, the included studies were defined as low- (1–3 stars), moderate- (4–6 stars), and high-quality (7–9 stars). Therefore, if the study obtained ≥4 stars, it was considered to have an above-moderate quality and, thus, was incorporated into our meta-analysis (31). Data extraction and quality assessment were conducted by two independent investigators (L.T. and Y.C.), and disagreements between them were resolved through negotiation with a third researcher (Z.S.).

Statistical Analysis

The statistical analyses were performed using Stata 15.1 software (Stata Corp, College Station, TX, USA). All of the tests were bilateral, and P values <0.05 were considered statistically significant, though P values >0.1 illustrated no heterogeneity among studies in the heterogeneity test (33). ORs, RRs, or HRs and their corresponding 95% CIs were considered to be the effect values of different infections on the risk of AS. The pooled OR and RR with their corresponding 95% CIs were used in case-control and cohort studies, respectively, to assess the association of infection with the risk of AS. We used the Q test and the I^2 statistic to detect heterogeneity among the studies. I^2 describes the percentage of total variation due to heterogeneity among studies rather than due to chance (34). In the presence of high heterogeneity (I^2>50%), the Dersimonian and Laird random effects model (REM) was adopted as the pooling method; otherwise, the Mantel-Haensze fixed effects model (FEM) was applied as the pooling method.

Subgroup analyses were performed based on adjusting for comorbidities, infection type, and infection site. In addition, considering that publication year, study location, sample size, definition of infection, and duration of follow-up (only in cohort studies) may affect between-study heterogeneity, subgroup analysis was also conducted based on these possible factors. Sensitivity analyses were performed to validate the stability of pooled ORs of case-control literature and pooled RRs of cohort literature by removing each individual study. In addition, we used the funnel plot and Begg's test to assess publication bias.

RESULTS

Literature Search and Study Selection

The flowchart of the literature search and study selection process is represented in **Figure 1**. Using three electronic databases and running the search strategy, a total of 4,584 potentially relevant articles were identified. In total, 1,358 duplicate articles were excluded. An additional 3,226 articles were excluded by screening for the title and abstract, leaving 24 articles for the full-text review. Screening *via* hand-searching found 1 relevant article. An additional 12 articles were excluded because they did not meet the inclusion criteria. Therefore, 13 articles that met the inclusion criteria were ultimately included (10, 11, 17–20, 22–28).

Study Characteristics

There were six case-control design articles (18, 22, 25–28) and seven cohort study design articles (10, 11, 17, 19, 20, 23, 24). It must be noted that one article involved two case-control studies (28). Therefore, we included 13 articles with 14 studies. The characteristics of the included studies, which were published from 2004 to 2020, are summarized in **Supplementary Tables 3–6**. The case-control studies included 1,296,239 participants; the cohort studies, 7,618,524 participants.

Seven studies were from Asia (11, 19, 22–25, 27), five, from Europe (10, 17, 26, 28), and two, from North America (18, 20). The mean NOS star score of the methodological quality of the studies was 7.9. Two articles (with three studies) obtained 5 stars (25, 28); two studies, 7 stars (17, 23); two studies, 8 stars (10, 26); and seven studies, 9 stars (11, 18–20, 22, 24, 27). The follow-up duration ranged from 3.6 to 34 years in the cohort studies. All studies involved both men and women (10, 11, 18–20, 22–28) except the study by Nielsen et al. (17). Nine studies defined the infection using clinical diagnosis (11, 17, 19, 20, 22–24, 26, 27), two studies (in the same article) defined the infection using self-report (28), and three studies defined the infection using laboratory tests in etiologic diagnoses (10, 18, 25). Thirteen studies reported one type of infection (10, 11, 19, 20, 22–28), and one study reported four types of infections (18). In the studies, except for those by Feng et al. and Yen et al., the risk estimates were adjusted for confounding variables, such as age, sex, urbanization, income, comorbidities, body mass index, educational attainment, smoking status, and alcohol consumption (10, 11, 17–20, 22, 24, 26–28).

Infection and the Risk of AS

Figures 2, **3** show the association between infections and the risk of AS in seven case-control studies (18, 22, 25–28) and seven cohort studies (10, 11, 17, 19, 20, 23, 24), respectively. There were significant heterogeneities among the seven case-control studies (P_Q<0.001. I^2 = 88.9%), and the seven cohort studies (P_Q<0.001. I^2 = 70.5%). Therefore, REM was used to pool the OR for case-control studies and RR for cohort studies. Our meta-analysis showed that, compared to the control group, the infection group was significantly associated with an increased risk of AS. The pooled OR calculated by REM was 1.46 (95% CI, 1.23–1.73) for the case-control studies, and the pooled RR was 1.35 (95% CI, 1.12–1.63) for the cohort studies.

Subgroup Analysis

Table 1 shows the subgroup analysis based on comorbidities (adjusted *vs*. unadjusted), infection type (bacterial *vs*. viral *vs*. other), and infection site (immune system *vs*. other).

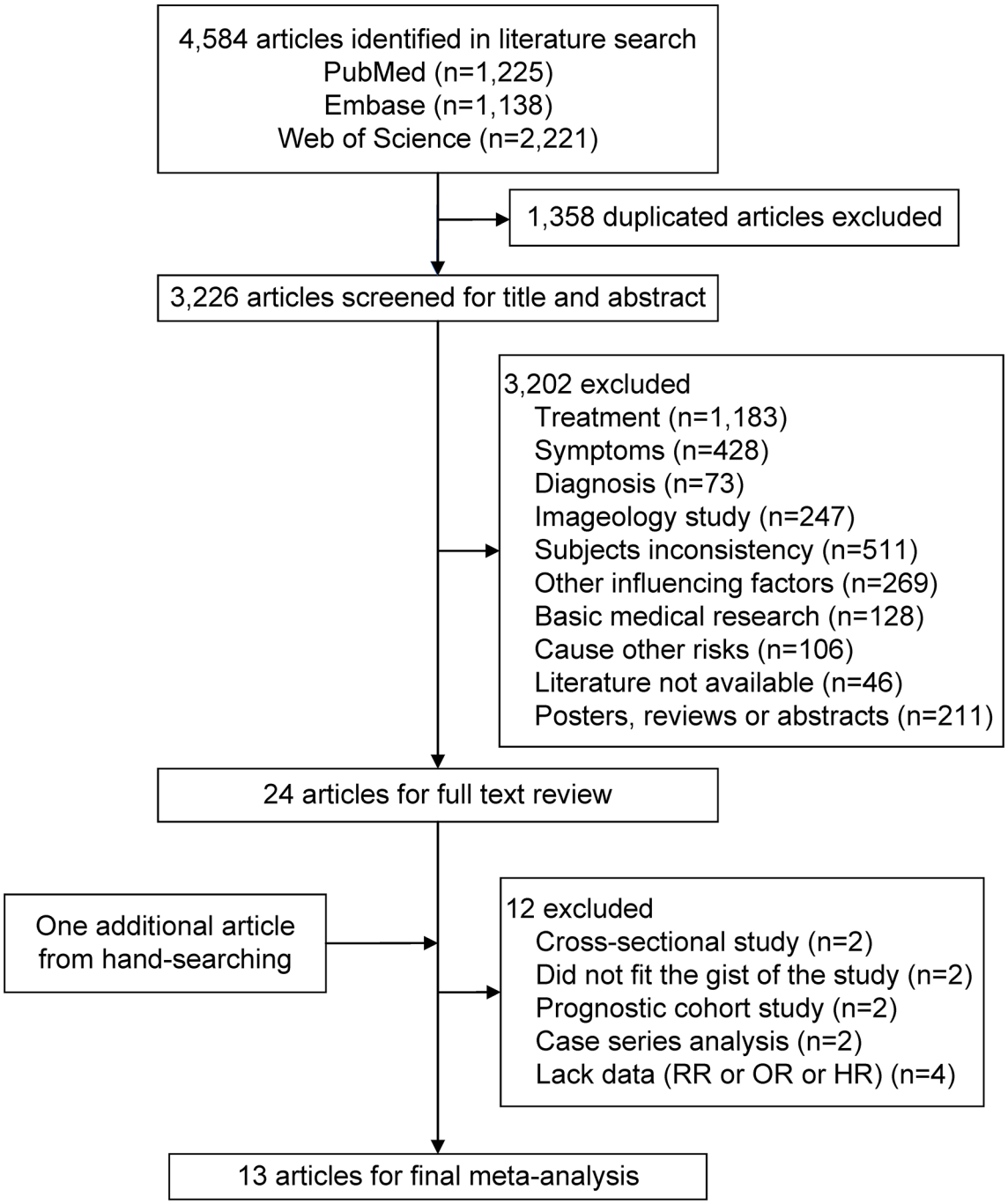

FIGURE 1 | Flowchart of the selection of studies included in this meta-analysis. HR, hazard ratio; OR, odds ratio; RR, relative risk.

In the case-control studies, our analysis showed no significant difference between adjusted comorbidities (OR=1.26, 95% CI, 0.94–1.70) and unadjusted comorbidities (OR=1.66, 95% CI, 1.35–2.03) (P=0.139). Similar results were reported with the infection type (bacterial, OR=1.31, 95% CI, 0.61–2.78; other, OR=1.40, 95% CI, 1.15–1.70; P=0.864) and the infection site (immune system, OR=1.46, 95% CI, 1.42–1.49; other, OR=1.27, 95% CI, 0.96–1.69; P=0.347).

In the cohort studies, our analysis showed no statistically significant difference between adjusted comorbidities (RR=1.39, 95% CI, 1.15–1.68) and unadjusted comorbidities (RR=0.86, 95% CI, 0.45–1.63; P=0.157). No differences were also found in the

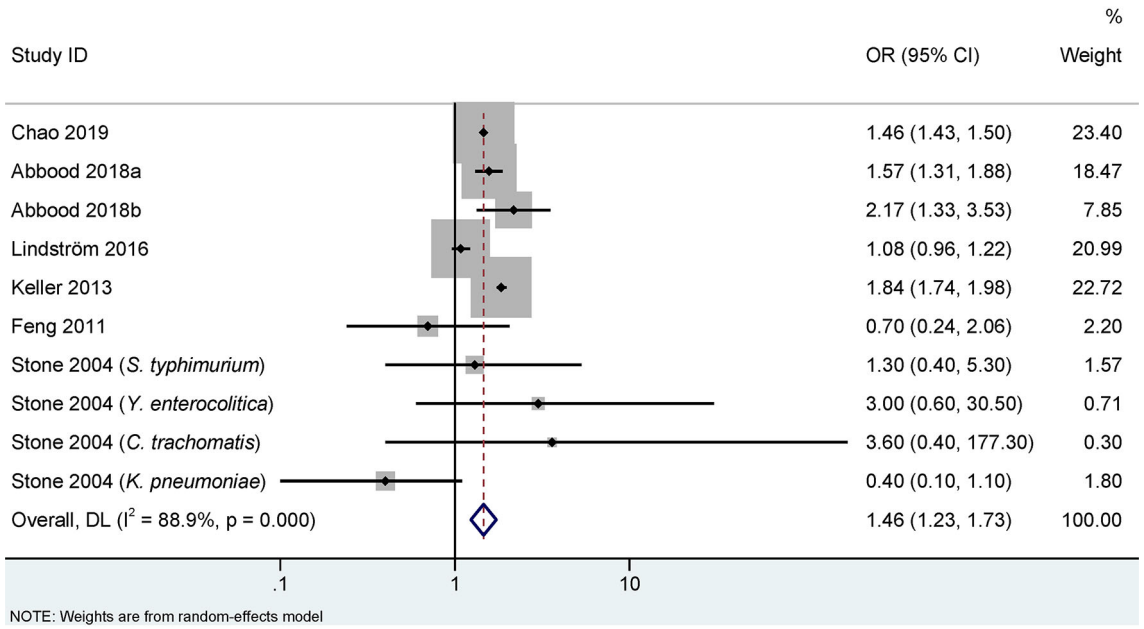

FIGURE 2 | Random effects meta-analysis of the association between infections and ankylosing spondylitis in case-control studies. CI, confidence interval; OR, odds ratio. a represents case-control study of self-reported ankylosing spondylitis; b represents case-control study of clinical recorded ankylosing spondylitis.

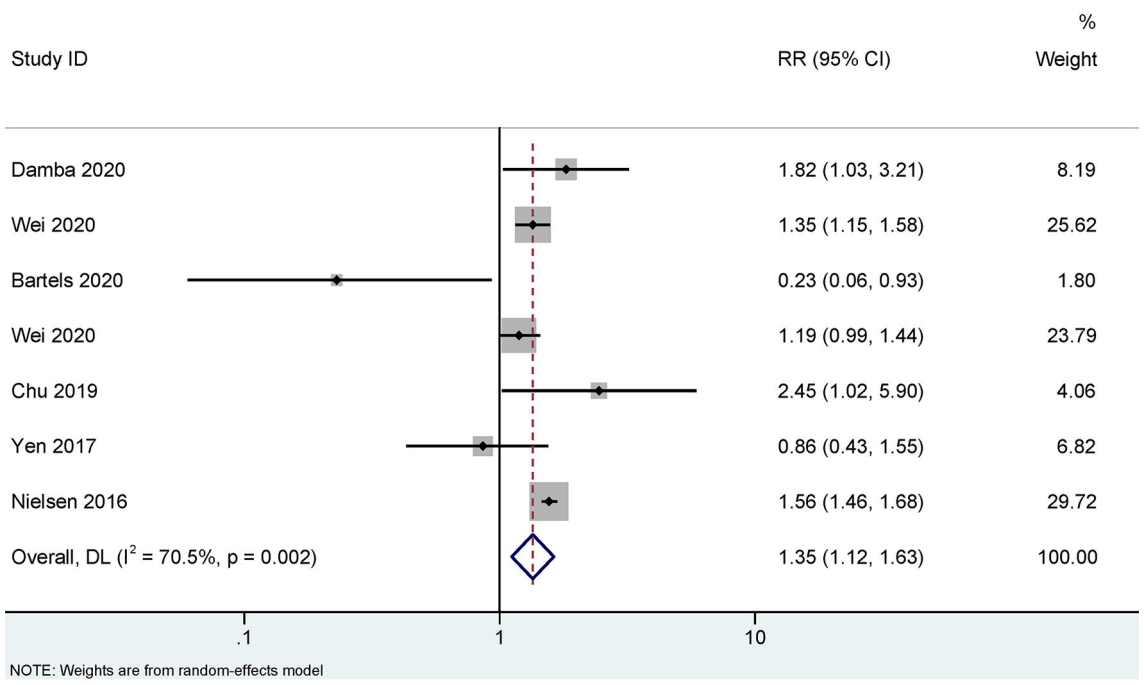

FIGURE 3 | Random effects meta-analysis of the association between infections and ankylosing spondylitis in cohort studies. CI, confidence interval; RR, relative risk.

TABLE 1 | The subgroup analyses of the association between infections and the risk of AS.

Subgroups	No. of items	OR/RR/HR (95% CI)	I^2 (%)	Chi-square test *P*-value
Case-control studies				
Comorbidities				0.139
Adjusted	2	1.26 (0.94–1.70)	95.7	
Unadjusted	8	1.66 (1.35–2.03)	45.8	
Infection Type				0.864
Bacterial	4	1.31 (0.61–2.78)	55.3	
Other*	6	1.40 (1.15–1.70)	82.7	
Infection Site				0.347
Immune system	2	1.46 (1.42–1.49)	0.0	
Other**	10	1.27 (0.96–1.69)	86.7	
Cohort studies				
Comorbidities				0.157
Adjusted	6	1.39 (1.15–1.68)	71.6	
Unadjusted	1	0.86 (0.45–1.63)	0.0	
Infection Type				0.766
Bacterial	2	0.70 (0.10–4.78)	87.4	
Viral	4	1.43 (1.22–1.66)	35.3	
Other*	3	1.44 (1.12–1.86)	74.0	
Infection Site				0.863
Immune system	2	1.27 (0.61–2.65)	66.0	
Other**	5	1.36 (1.11–1.67)	76.6	

AS, ankylosing spondylitis; CI, confidence interval; HR, hazard ratio; OR, odds ratio, RR, relative risk.
**Other types of infection include fungi, chlamydia, and mycoplasma.*
***Other sites of infection include the digestive system, respiratory system, and genitourinary system.*

infection type (bacterial, RR=0.70, 95% CI, 0.10–4.78; viral, RR=1.43, 95% CI, 1.22–1.66; other, RR=1.44, 95% CI, 1.12–1.86; *P*=0.766) and infection site (immune system, RR=1.27, 95% CI, 0.61–2.65; other, RR=1.36, 95% CI, 1.11–1.67; *P*=0.863).

We also conducted subgroup analysis based on publication year, study location, sample size, definition of infection, and duration of follow-up. As shown in **Supplementary Table 7**, the results showed no significant difference between those subgroups in the case-control studies, which indicated that these above factors were not the source of heterogeneity in case-control studies. In the cohort studies, only for definition of infection, there was a significant deference between clinical diagnosis (RR=1.39, 95% CI, 1.19–1.63, I^2 = 62.3%) and basic medical experiment (RR=0.23, 95% CI, 0.06–0.91, I^2 = 0%, *P*=0.010). Definition of infection might be one of the sources of heterogeneity in the cohort studies.

Sensitivity Analysis and Publication Bias Detection

As shown in **Figure 4**, the results of sensitivity analysis showed that no individual study significantly influenced the pooled OR in the case-control studies. However, the study by Nielsen et al.

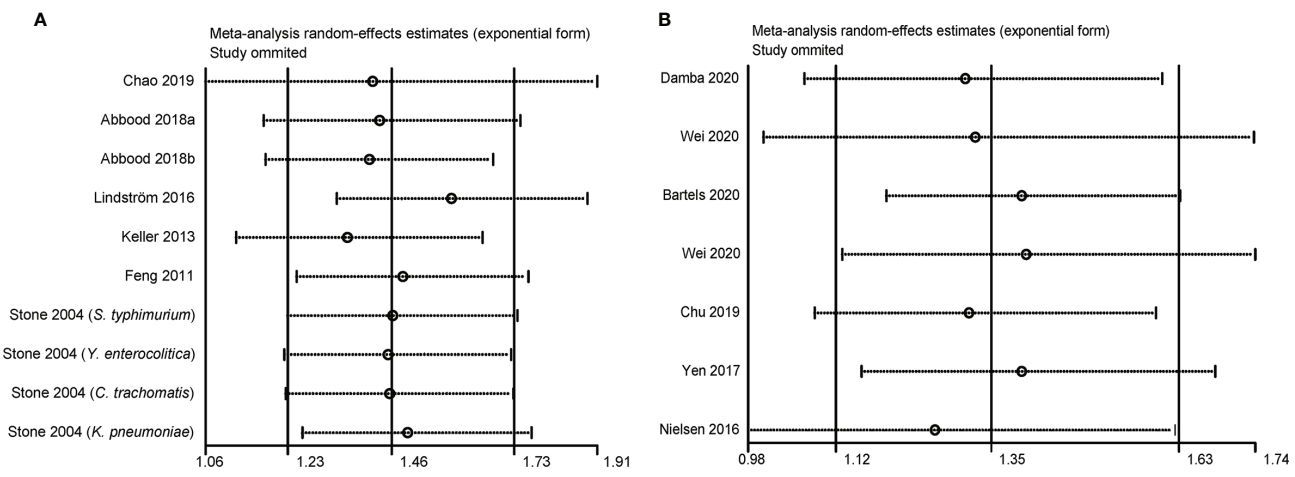

FIGURE 4 | Sensitivity analysis of the association between infections and ankylosing spondylitis. **(A)** Case-control study; a represents case-control study of self-reported ankylosing spondylitis; b represents case-control study of clinical recorded ankylosing spondylitis; **(B)** Cohort study.

affected the pooled RR in the cohort studies. The pooled RR was 1.26 (95% CI, 0.98–1.62; I^2 = 58.6%) by omitting this study.

The funnel plots for estimating publication bias were roughly symmetrical for the case-control (**Figure 5A**) and cohort studies (**Figure 5B**). No publication bias was detected by Begg's test for the case-control (P=0.721) and cohort studies (P=0.368).

DISCUSSION

To the best of our knowledge, this meta-analysis is the first study to investigate the association between infections and the risk of developing AS. The current meta-analysis included seven case-control studies with 1,296,239 participants and seven cohort studies with 7,618,524 participants. The results of this study confirm that infections play an important role in the risk of AS. Determining the effect of infections on AS would be beneficial for the identification of those who are at higher risk of AS as reasonable preventive intervention can be conducted for this population, enabling a far-reaching significance for the prevention of AS.

The results showed that infections are associated with an increased risk of AS in both the case-control and cohort studies. Our findings are consistent with most of the studies, including the four studies from Asia (19, 22, 24, 27), three studies from Europe (17, 28), and one study from North America (20). However, the study by Bartels et al. showed opposite results (10), in that previous *Helicobacter pylori* (*H.pylori*) infection may reduce the risk for developing AS. Another study found that *H. pylori* infection was eradicated in more than 80% of cases in the same cohort as that of Bartels et al. (35), indicating that *H. pylori* leaves a protective potential for the development of AS later in life when it is eradicated (10). Furthermore, the microbiota in the gastrointestinal tract changes after the eradication of *H. pylori*, which may have an impact on AS development (36, 37).

Our subgroup analyses showed that there was an association between infection and the risk of AS after adjusting for comorbidities in the cohort studies, which suggested that the comorbidities are a remarkably important confounding factor in cohort studies, and that we must control and adjust it. However, an association between infection and the risk of AS was found in the case-control studies without adjusted comorbidities. This is due to the nature of the case-control study design. As one of the matching factors of case-control studies, comorbidities may be matched in the design stage, cancelling the need to adjust for comorbidities in the statistical analysis stage.

With regards to the infection types, we did not observe that bacterial infections contribute to the risk of AS. In case-control studies, only the study by Keller et al. showed that there is an association between AS and a prior diagnosis of chronic periodontitis, which is characterized by an oral bacterial infection (22). This may be because rheumatic diseases and chronic periodontitis share pathogenic factors, including a dysfunction of inflammatory mechanisms and an imbalance of proinflammatory and anti-inflammatory cytokines (22, 38–40). In the cohort studies, the study by Bartels et al. showed that *H. pylori* may be a protective factor for AS (10). The study of Nielsen et al. also showed that bacterial infection is associated with the development of AS in the general population (17). More cohort studies are needed to verify whether bacterial infection causes AS. The result of the subgroup of other infection types was that other infection types are associated with AS in both the case-control and cohort studies. In our analysis, other types of infection included fungal, chlamydia, and mycoplasma. The pathogenesis of AS due to other types of infection is far from clear. For example, one study suggested that AS could be induced after exposure to *Candida albicans* through a T cell-driven model towards Th17 responses (11). Another study suggested that *Mycoplasma pneumoniae* has a significant impact on immune cells and the immune system of the host, including polyclonal activation of T and B cells and the secretion of related cytokines (24), leading to a breakdown of immune-tolerance. In addition, subgroup analysis indicated that viruses play an important role in the risk of AS in the cohort studies. One study suggests that viruses (e.g., human

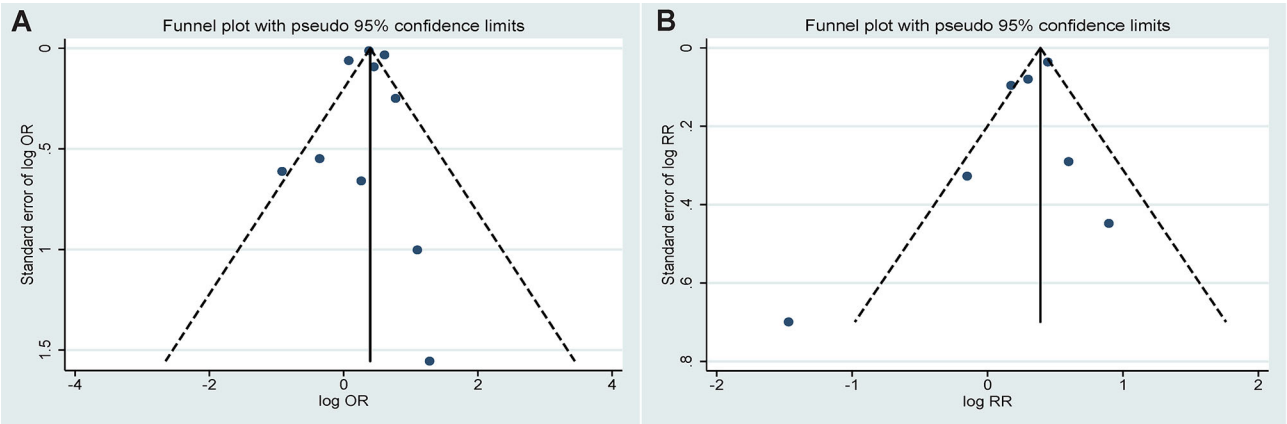

FIGURE 5 | Funnel plots corresponding to the random effects meta-analysis of the association between infections and ankylosing spondylitis. **(A)** Case-control study (P=0.721 by Begg's test); **(B)** Cohort study (P=0.368 by Begg's test).

papillomavirus) might lead to inflammatory or immune-mediated disease by activating the pathogenic IL-23/IL-17 axis, resulting in elevated serum levels of Th17 cells, IL-17, and IL-23, and the imbalance of IL-17A/IL-23 cytokines (19). In the subgroup analysis of infection sites, we found that the infection of the immune system was significantly associated with the risk of AS in the case-control studies. Some immune organs, such as the tonsils, are involved in allergens tolerance by generating allergen-specific $FOXP3^+$ regulatory T cells, suggesting that they are critical in the development of immune-tolerance (41). Some studies postulated that the alteration of immune tolerance in patients with tonsillitis might lead to the inflammatory disorders in autoimmune arthritis, including AS; therefore, tonsillitis might be aggravated by spondylitis, leading to the diagnosis of AS (26, 27). In addition, the higher risks of AS among infected people might be explained by HIV-induced antigen-driven immune responses (42), T cell imbalance (43), and molecular mimicry located between HIV protein and self-antigens (44). For the cohort studies, the infections in other sites were significantly associated with the risk of AS, which indicates that AS might be triggered by respiratory tract infections and genitourinary system infections. The pathogenesis of AS caused by the infection in the genitourinary system is mixed. In one of them, human papillomavirus in the genitourinary system might lead to AS by activating the IL-23/IL-17 axis (19). For the respiratory system, *Klebsiella pneumoniae* might lead to a decrease in the number of specific T cells, which could reflect an insufficient in the host's defense against *Klebsiella*, thereby allowing AS to be affected by bacterial antigens that reach the joint (18). In our study, we found that some design types were meaningful and some were not for the same subgroups, which might be related to the small number of included articles or the large heterogeneity between the included studies.

This meta-analysis has the following strengths: pooled effect values were analysed according to the different study design, and we grouped the studies according to the types and sites of infection to determine whether these factors were associated with the risk of AS. This study included analytical epidemiological studies to determine the risk of AS, and the sample size was large. The included studies were adjusted for potential confounding variables, which improved the accuracy of risk estimates.

However, some limitations have affected the current study. First, although heterogeneity was explored *via* subgroup analysis, it was still very high, which may be related to age, sex distribution of participants, definition of infection, diagnosis of AS, etc. The subgroup analysis suggested that definition of infection was one of sources of heterogeneity in the cohort studies, which indicated that the possible disagreement between measurement methods might be a source of misclassification. But heterogeneity within subgroups remained high. In addition, although we extracted the definition of AS, only two articles declared that the diagnosis of AS was based on the Amor criteria (18, 25). As most of articles were retrospective, the International Classification of Diseases codes for the diagnoses of AS were based on records made by physicians and hospitals rather than a prospective clinical setting; thus, we could not set uniform criteria for the definition of AS, which also may result in heterogeneity. Second, this meta-analysis included only English- and Chinese-language articles, and qualified articles in other languages were not included in the analysis, which might have affected the pooled estimated value. Third, our pooled effect is affected by the study by Nielsen et al. in the cohort studies. However, the study by Nielsen et al. has the largest weight when synthesizing RRs across studies, because of its large sample size, narrow confidence interval, and high quality. When the study by Nielsen et al. was omitted in the sensitivity analysis, the pooled effect estimate was affected by some low-quality studies due to the increased weights. Thus, more large cohort studies are recommended in the future to assess the impact of infections on the risk of AS.

In conclusion, this meta-analysis confirms that there is an association between infections and an increased risk of AS, although the included studies suffered from high heterogeneity. As much as the mechanism of infection and the effect of bacterial and viral infections on AS has not yet been determined, further studies, particularly more higher quality prospective cohort studies and case-control studies, are required to verify that there is a true cause-and-effect relationship between infections and the risk of developing AS.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

GD and XZ designed the study protocol. XZ, XS, and JY conducted the literature search. XZ, AZ, and GD retrieved and selected the articles. LT, YC, and ZhengS conducted data extraction. XZ and ZheS performed the statistical analysis of the data. XZ, ZheS, and GD wrote the manuscript draft. GD and ZhengS supervised the study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.768741/full#supplementary-material

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