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Research article

T-bet^{+}ILC3 in peripheral blood is increased in the ankylosing spondylitis with high disease activity

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ABSTRACT

Objective: Ankylosing spondylitis (AS) is a chronic autoimmune disease characterized by systemic inflammation, often resulting in fusion of the spine and peripheral joints. This study aimed to investigate the role of innate lymphoid cells (ILCs) in AS patients with high disease activity.
Methods: Blood samples were collected from healthy controls and AS patients categorized by high or low disease activity. Systemic inflammation was quantified through C-reactive protein (CRP) levels and erythrocyte sedimentation rate (ESR), alongside disease activity scores such as Ankylosing Spondylitis Disease Activity Score(ASDAS) and Bath Ankylosing Spondylitis Disease Activity Index(BASDAI). The levels of different ILC subsets and the expression of T-box transcription factor 21 (T-bet) and retinoic-acid-receptor-related orphan receptor gamma (RORγt) in peripheral blood were analyzed via flow cytometry. Additionally, 24 cytokines in plasma were measured using a Luminex liquid suspension chip.
Results: The proportion of total ILCs and the distribution of ILC subsets in peripheral blood varied with AS disease activity scores. Specifically, the frequencies of total ILCs and ILC3s were significantly elevated in AS patients with high disease activity (AS-HA). The frequency and absolute number of ILC3s showed a positively correlation with disease severity scores. Furthermore, T-bet^{+}ILC3s were significantly increased in the AS-HA group. Plasma levels of IL-17A and IFN-γ were positively correlated with the frequency of circulating-ILC3 in AS patients.
Conclusions: These findings highlight the critical role of T-bet^{+}ILC3s in the inflammatory process of AS, suggesting their potential as a therapeutic target for managing AS disease.

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1. Introduction

Ankylosing spondylitis (AS), also known as axial spondylarthritis (axSpA), is a chronic autoimmune disease characterized by systemic inflammation. It primarily affects the spine, peripheral joints, and entheses, ultimately leading to abnormal bone remodeling and ankylosis (bone fusion) [1]. Common extra-articular manifestations of AS include uveitis (inflammation of the eye), psoriasis (skin condition characterized by patches of red, itchy, and scaly skin), and inflammatory bowel disease (IBD) [2].

It is well known that interleukin-17A (IL-17A) plays a crucial role in driving inflammation in AS by promoting the recruitment of neutrophils and enhancing the production of pro-inflammatory cytokines such as TNF-α and IL-6. Treatments targeting IL-17A, such as Secukinumab, have demonstrated significant efficacy in reducing inflammation and improving mobility in AS patients [3]. While Th17 cells were once thought to be the primary source of IL-17, recent research has shown that innate lymphoid cells (ILCs) also contribute significantly to IL-17 production [4–6]. ILCs are a group of innate immune cells that do not possess lineage markers for T cells, B cells, myeloid and dendritic cells, monocytes and macrophages, mast cells, or stem cells [7–9]. ILCs resemble the functions of T cells and develop from common lymphoid progenitors, but different transcription factors suppress lymphocyte fates and lead to the generation of various subsets of ILCs [10,11]. Based on their functions, ILCs can be broadly classified into helper type ILCs and cytotoxic ILCs. Helper type ILCs include type 1 ILCs (ILC1s), type 2 ILCs (ILC2s), and type 3 ILCs (ILC3s), which correspond to innate counterparts of $CD4^{+}$T helper Th1, Th2, and Th17 cells, respectively [11]. Recent studies have established a close association between ILC3s and AS [12,13]. For instance, data from joints tissues and entheses of AS patients revealed a marked accumulation of ILC3s, correlating with local inflammatory markers. Additionally, peripheral blood analyses in patients with spondylarthritis (SpA) indicated that elevated ILC3 levels align with heightened IL-17 production and disease activity [14]. Expansion of ILC3s has also been observed in inflamed synovial fluid, bone marrow, intestinal tract, and peripheral blood of AS patients [13], and the plasticity of ILCs has been implicated in intestinal and joint inflammation in AS [15]. However, the specific role of ILC3s in AS is still not fully understood.

In this study, we investigated the population and distribution of ILCs, particularly ILC3s, in the peripheral blood of AS patients with low or high disease activity (AS-LA and AS-HA, respectively). Our findings revealed that ILC3 subsets are strongly correlated with the activation of AS, and T-bet^{+}ILC3s likely play a significant role in AS disease relapse. Our study highlights the importance of understanding the role of ILCs in AS pathogenesis and provides new insights into potential therapeutic targets for the management of AS disease. Specifically, the findings suggest that targeting T-bet^{+} ILC3s or modulating their production of IL-17 could be promising strategies for mitigating disease progression and inflammation in AS.

2. Materials and methods

1 Human Subjects

All subjects in this study were recruited from Shanghai Guanghua Hospital of Integrated Traditional Chinese and Western Medicine between March 2020 and December 2021. Participants were identified through outpatient clinics and inpatient wards, and eligibility was determined based on inclusion and exclusion criteria evaluated by trained clinicians. The study included 42 patients with ankylosing spondylitis (AS) as well as 9 healthy controls. The inclusion criteria for AS patients were as follows: (1) Meeting the 1984

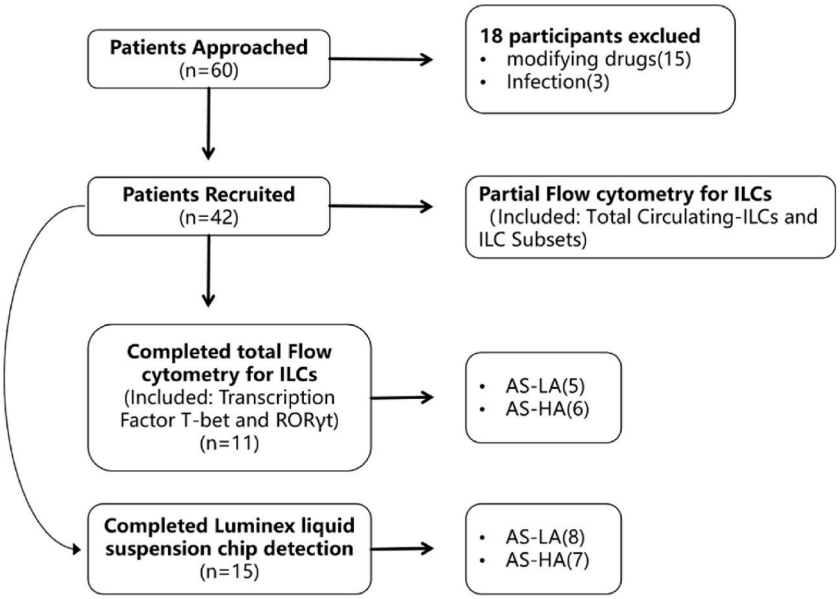

Fig. 1. Study flow diagram.

modified New York (mNY) criteria for AS [16]; (2) Aged between 18 and 65, regardless of gender; (3) No treatment with AS-modifying drugs such as bioinhibitors for three months. The exclusion criteria were as follows: (1) Patients with diarrhea, infection, trauma, or other inflammatory diseases in the past month; (2) Patients diagnosed with other rheumatic autoimmune diseases or immunodeficiency syndrome, such as inflammatory bowel disease, psoriasis, active uveitis, etc; (3) Patients with asthma, chronic obstructive pulmonary disease (COPD), diabetes, serious cardiovascular and cerebrovascular diseases, tumors, active gastrointestinal diseases, or any other significant underlying conditions, as well as pregnancy and breastfeeding. Additionally, subjects who the investigator deemed not appropriate for inclusion in the study were also excluded. Informed consent was obtained from all participants. The study flow diagram can be found as Fig. 1.

2 Study Protocol

Peripheral blood (PB) samples were collected from all subjects, including 42 AS patients and 9 health control (HC), for analysis using flow cytometry. Approximately 5 mL of blood was drawn from each participant to ensure sufficient volume for consistent and reproducible analysis. Levels of C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) were measured in AS patients. Disease activity scores, including ankylosing spondylitis disease activity score (ASDAS)-CRP, ASDAS-ESR, and bath ankylosing spondylitis disease activity index (BASDAI) scores, were evaluated. 22 AS patients were categorized into the low disease activity group (AS-LA, ASDAS-CRP$<$2.1) and 20 were categorized into the high disease activity group (AS-HA, ASDAS-CRP$\geq$2.1) [17,18]. The experimental protocol was approved by the Ethics Committee of Guanghua Hospital (Ethics Approval No. 2020-K-45). All participants were provided with written information about study, and the study processed only after obtaining signed informed consent. Minors were excluded from participation.

3 Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

Venous blood samples from AS patients and HC were collected into Li-heparin-treated tubes to prevent clotting and ensure the preservation of cell integrity, which is critical for subsequent analyses such as flow cytometry. Peripheral blood mononuclear cells (PBMCs) were isolated from 1 mL of blood using Ficoll-Hypaque density gradient centrifugation at 20 °C for 20 min at 500 g. The PBMCs were subsequently analyzed for ILC subsets using flow cytometry [19]. All analyses were performed on freshly prepared samples.

4 Flow Cytometry for ILCs

Freshly isolated PBMCs were resuspended in phosphate buffered saline (PBS) and incubated with a live/dead cell marker(Fixable Viability stain 520 BD 564407) for 10 min at 4 °C. Cells were washed and resuspended in fluorescence-activated cell sorting(FACS) buffer containing 2 % fetal bovine serum (FBS), 2 mM ethylenediaminetetraacetic acid (EDTA) in PBS. Cells were first incubated with 1:50 dilution human Fc Block for 10 min at 4 °C and then stained with antibodies mixture targeting CD45, CD127, CD117, CRTH2, and lineage markers (TCRγ/δ, TCRα/β, CD3, CD19, CD14, CD16, CD94, CD123, CD34, CD303 and FcεRI) for 30 min at room temperature, as listed in Table 1 [19]. After washing, cells were centrifuged and resuspended in FACS buffer. Intracellular staining was performed using the BD Human Foxp3 Buffer kit, following the manufacturer's instructions. Cells were stained for intracellular transcription factors (T-bet, RORγt), incubated for 30 min at 4 °C, washed twice with staining buffer and analyzed on a BD Symphony (BD Biosciences). Flow cytometry data was processed using FlowJo software (FlowJo LLC).

Table 1
Antibodies for flow cytometry.

Antibodies	SOURCE	Clone
Fc Receptor Blocking Solution	BioLegend	
Live/Dead APC-Cy7	ThermoFisher	
Anti-human CD3 antibody	Biolegend	UCHT1
Anti-human CD14 antibody	Biolegend	M5E2
Anti-human CD16 antibody	eBioscience	eBioCB16
Anti-human CD19 antibody	Biolegend	HIB19
Anti-human FcεR1 antibody	eBioscience	AER-37
Anti-human CD34 antibody	Biolegend	581
Anti-human CD94 antibody	Biolegend	DX22
Anti-human CD123 antibody	Biolegend	6H6
Anti-human CD303 antibody	Biolegend	201A
Anti-human TCR ab antibody	Biolegend	IP26
Anti-human TCR gd antibody	Biolegend	B1
Anti-human CD45 antibody	Biolegend	2D1
Anti-human CD127 antibody	BD Bioscience	HIL-7R-M21
Anti-human CD117 antibody	Biolegend	104D2
Anti-human CD294 (CRTH2) antibody	BD Bioscience	BM16
Anti-human RORγt antibody	BD Bioscience	Q21-559
Anti-human T-bet antibody	eBioscience	eBio4B10

ILCs were identified as $CD45^+$ lineage-negative (Lin-) cells expressing the α-chain of the IL-7 receptor (CD127). Lin markers included CD3, CD14, CD16, CD19, CD34, CD94, CD123, CD303, FcεR, TCRα/β, and TCRγ/δ [19]. The gating strategy for identifying circulating ILC subsets is shown in Fig. 1a. The analysis focused on the proportions and phenotypes of ILCs subsets. ILC1 and ILC3 were defined as $CD117^-CRTH2^-$and $CD117^+CRTH2^-$cells, respectively, while $CRTH2^+$ cells were classified as ILC2. To further investigate the function of amplified ILC3 in AS, we analyzed the transcription factors T-bet and RORγt in ILC subsets.

5 Luminex Liquid Suspension Chip Detection of Chemotactic and Inflammatory Cytokines

To assess the potential function of ILC3 in peripheral blood, 24 chemotactic and inflammatory cytokines in plasma were measured using a Luminex liquid suspension chip.Samples were obtained from the AS-HA and AS-LA groups. The analysis was conducted by Wayen Biotechnologies (Shanghai, China) using the Bio-Plex Pro Human Cytokine Grp I Panel 27-plex kit, following the manufacturer's guidelines. Plasma samples were incubated in 96-well plates containing microbeads for 1 h followed by incubation with a detection antibody for 30 min. Streptavidin-PE was subsequently added to each well for 10 min, and cytokine levels were measured using the Bio-Plex MAGPIX System(Bio-Rad).

6 Statistical Analysis

Statistical analyses were performed using GraphPad Prism software (version 8.0.2; GraphPad Software Inc., CA, USA). Data are presented as mean ± standard deviation (SD). The normality of data distribution was assessed using Shapiro-Wilk test. For data following a normal distribution, difference between two groups were evaluated using an unpaired Student's t-test, while differences among three groups were analyzed using one-way analysis of variance (ANOVA). Post-hoc pairwise comparisons were performed using the Least Significant Difference (LSD) test. For data not following a normal distribution, the Kruskal-Wallis's test was applied. Bivariate linear regression analysis was conducted to evaluate correlations. A p-value of less than 0.05 was considered statistically significant.

3. Result

1 Patient Characteristics

A total of 22 AS patients were identified as having low disease activity (AS-LA), while 20 AS patients were identified as having high disease activity (AS-HA). There was no significant difference in age observed among the three groups. However, the AS-HA group exhibited significantly higher levels of CRP, ESR, ASDAS, and BASDAI scores compared to the AS-LA group (as shown in Table 2).

2 Increased Proportion of Total Circulating ILCs in AS patients with High Disease Activity

To assess differences in circulating total ILCs and their subpopulations among AS-HA, AS-LA and HC group, we measured the percentage of peripheral ILCs within $CD45^+$ cells, subpopulation of ILCs and their absolute counts. The flow cytometry gating strategies for identifying peripheral blood ILC subsets is depicted in Fig. 2a. Initially, we compared total ILC levels between HC and AS patient. The AS patients are future divided into AS-HA and AS-LA groups. The result showed that there were no significant differences in total ILC percentage or absolute counts between AS patients and HC. However, the ILC/$CD45^+$ ratio and absolute counts of ILCs were markedly elevated in the AS-HA group compared to both the HC and AS-LA groups (Fig. 2b and c). These findings suggest a significant increase in circulating-ILCs in AS patients with high disease activity.

3 ILC3 Subsets Was Significant Amplification in AS Patients with High Disease Activity.

To further investigate ILC subset distribution, we analyzed the frequencies and absolute counts of ILC subsets in AS patients. As

Table 2
Participant characteristics.

Characteristic	AS-LA	AS-HA	HC	*P* value
N	22	20	9	–
Gender,F/M	5/17	4/16	3/6	0.731
Age, mean (SD), years	40.00(13.09)	41.30(13.71)	33.56(9.76)	0.318
CRP,mean(SD),mg/L	2.93(3.53)	14.33(14.54)	-	0.001**
ESR,mean(SD),mm/h	9.14(8.08)	33.80(29.23)	-	***
ASDAS-CRP,mean(SD)	1.31(0.49)	3.02(0.86)	-	***
ASDAS-ESR,mean(SD)	1.40(0.51)	3.05(1.01)	-	***
BASDAI, mean(SD)	1.99(1.19)	3.74(1.26)	-	***

The characteristics of patients with AS-LA, AS-HA, and HC were assessed by one-way ANOVA. The differences in CRP, ESR, ASDAS-CRP, ASDAS-ESR and BASDAI between AS-LA and AS-HA were evaluated using an independent-samples *t*-test. $*P < 0.05$,$**P < 0.01$,$***P < 0.001$. AS-LA, AS patients with low disease activity; AS-HA, AS patients with high disease activity; CRP, C reactive protein; ESR Erythrocyte sedimentation rate; ASDAS, Ankylosing Spondylitis Disease Activity Score; BASDAI Bath Ankylosing Spondylitis Disease Activity Index.

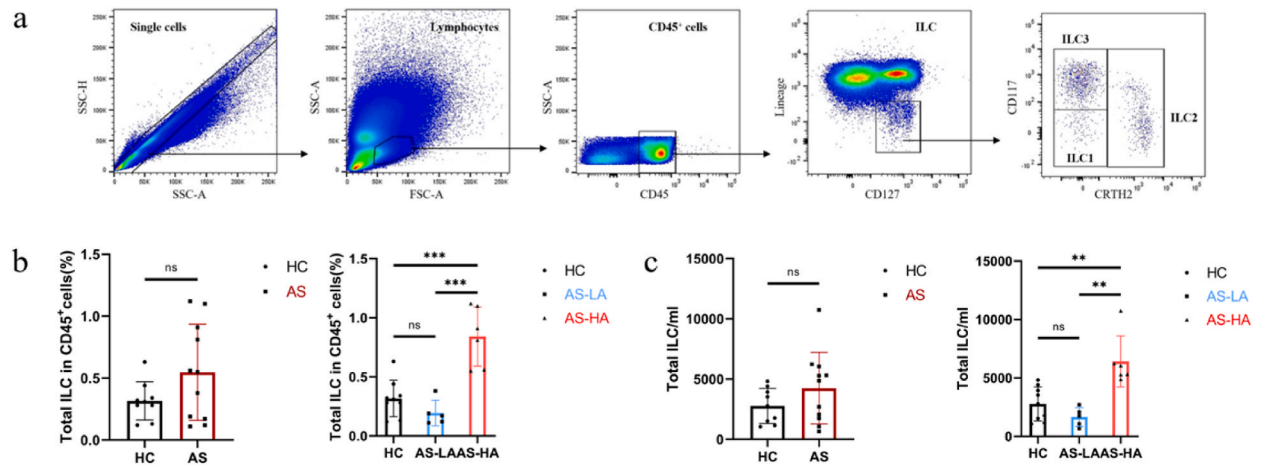

Fig. 2. Total Circulating-ILC Proportion was increased in patients with AS-HA.
a. Flow cytometry gating strategies for the identification of peripheral blood ILC subsets. ILCs were defined as CD45+Lin-(including CD3, CD14, CD16, CD19, CD34, CD94, CD123, CD303, FcεR, TCRα/β, and TCRγ/δ) CD127+cells. CD117(c-kit) and CRTH2 were used to determine the distribution of ILC subsets: ILC1($CD117^{-}CRTH2^{-}$), ILC2($CRTH2^{+}$) and ILC3($CD117^{+}CRTH2^{-}$); b. Histograms represent the percentage of peripheral ILCs in $CD45^{+}$cells in the three groups(HC = 9, AS-LA = 5, AS-HA = 6); c. Histograms represent the absolute counts of total ILCs in the three groups. Data are shown as means ± SD. Two groups was assessed using an unpaired Student's t-test, while differences between three groups were compared using ANOVA. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. *ns*, not significant.

shown in Fig. 3, ILC3 constituted the majority of total circulating ILCs ($Lin^{-}CD45^{+}CD127^{+}$) in AS patients with. Compared to HC, the ILC3 frequency and counts were significantly increased in AS patients, whereas ILC1 and ILC2 proportions within total ILCs showed no significant differences (Fig. 2a and b). Compared to HC and AS-LA group, the frequency of ILC3 among total ILCs was significantly higher, while ILC1 and ILC2 proportions were lower in the AS-HA group (Fig. 3c). Similarly, the absolute counts of ILC3 were significantly increased in AS-HA compared to HC and AS-LA, with no significant changes observed in ILC1 or ILC2 subsets (Fig. 3d). These findings indicate that increase in ILC3 in AS patients is driven by ILC3 expansion, rather than a reduction in ILC1 and ILC2 subsets.

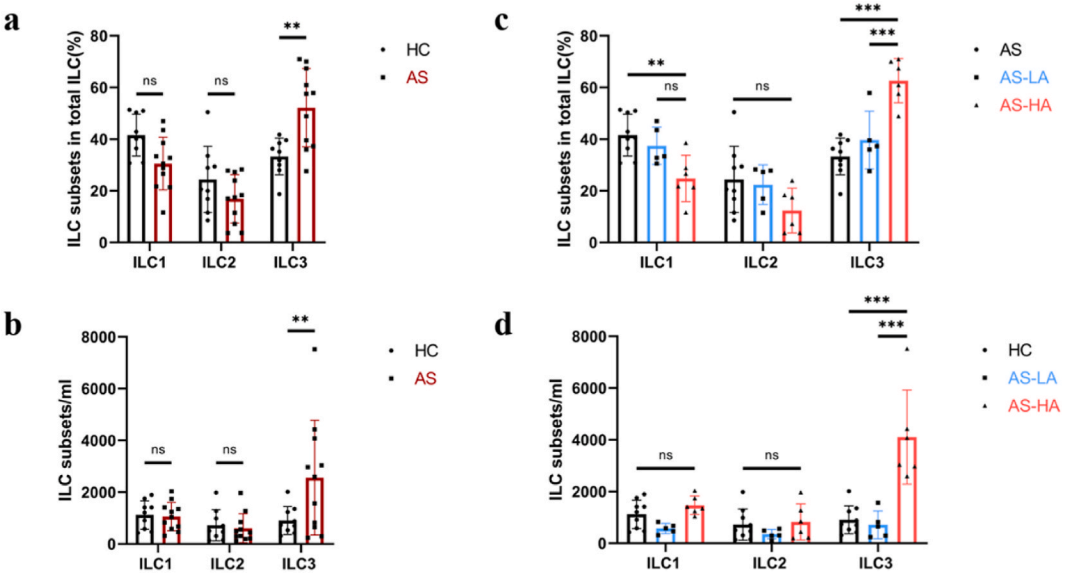

Fig. 3. Amplification of ILC3 subsets in AS-HA.
a. Histograms represent the percentage of each ILC subsets in total ILCs between AS(n = 11) and HC(n = 9); b. Histograms represent the absolute counts of each ILCs subsets between AS and HC; c. The percentage of each ILC subsets in total ILCs in the three groups(HC = 11, AS-LA = 5, AS-HA = 6); d. The absolute counts of each ILCs subsets in the three groups. Data are shown as means ± SD. Post-hoc pairwise comparisons were performed using the Least Significant Difference (LSD) test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.*ns*, not significant.

4 Abnormal Distribution of ILC Subsets Correlated with Disease Activity

To determine the relationship between ILCs subsets and disease activity in AS, we conducted a correlation analysis between ILC1, ILC2, and ILC3 populations and disease activity indicators, including CRP, ESR, ASDAS-CRP, ASDAS-ESR, and BASDAI. As shown in Fig. 4a, the frequency of ILC1/ILCs was significantly negatively correlated with inflammatory indicators (CRP, ESR) and disease activity scores (ASDAS-CRP, ASDAS-ESR) in AS patients. In contrast, the frequency of ILC2/ILCs showed no correlation with inflammatory indicators and disease activity scores (Fig. 4b). Conversely, the frequency of ILC3/ILCs was significantly positively correlated with disease activity scores (ASDAS-CRP, ASDAS-ESR, BASDAI) (Fig. 4c). The results suggest that abnormal distribution of ILCs subsets in AS patients correlates closely with disease activity. ILC3 and ILC1 likely play distinct roles in modulating disease activity, with changes in their populations potentially influencing disease progression.

5 T-bet^{+}ILC3 Exists in the Peripheral Blood of AS Patients with High Disease Activity

Previous studies have demonstrated that the T-bet gene is upregulated in ILC1, while the RORγt gene is upregulated in ILC3. Furthermore, potential transdifferentiation between ILC1 and ILC3 subpopulations has been suggested. Our results revealed a significant increase in the ILC3 population alongside a decrease in the ILC1 population in AS patients with high disease activity(Fig. 3c). This raises the question: could transdifferentiation between ILC1 and ILC3 be responsible for this shift in cell distribution, leading to the observed expansion of ILC3? To address this, we examined the expression of key genes associated with ILC1(T-bet) and ILC3 (RORγt) across the three groups. Patients characteristics are summarized in supple. Table 1. As shown in Fig. 5a, the expression levels of T-bet and RORγt in CRTH2^{-} ILC were analyzed in HC, AS-LA and AS-HA groups. The results indicated that the proportion of T-bet^{+}ILC3 was significantly increased in the AS-HA group compared to both HC and AS-LA groups, whereas no significant differences were observed in the proportions of T-bet^{+}ILC1, RORγt^{+}ILC1 and RORγt^{+}ILC3 among the three groups (Fig. 5a and b). A similar trend was observed in the absolute counts of T-bet^{+}ILC3 in AS-HA patients (Fig. 5c). The findings suggest the existence of a novel ILC3 subpopulation, T-bet^{+} ILC3, in the peripheral blood of AS patients with high disease activity. This subpopulation, which appears to be an intermediate form between ILC1 and ILC3, may be primarily responsible for the observed expansion of ILC3 cells in these patients. As shown in Fig. 4, our results further indicate the contribution of ILC3 is positively associated with inflammation activity in AS. Thus, we propose that T-bet^{+} ILC3 may play a pivotal role in the inflammation and disease relapse in AS.

6 T-bet^{+}ILC3 Correlates Significantly with Disease Activity in AS and Is Associated with Increased Release of IL-17A and IFN-γ

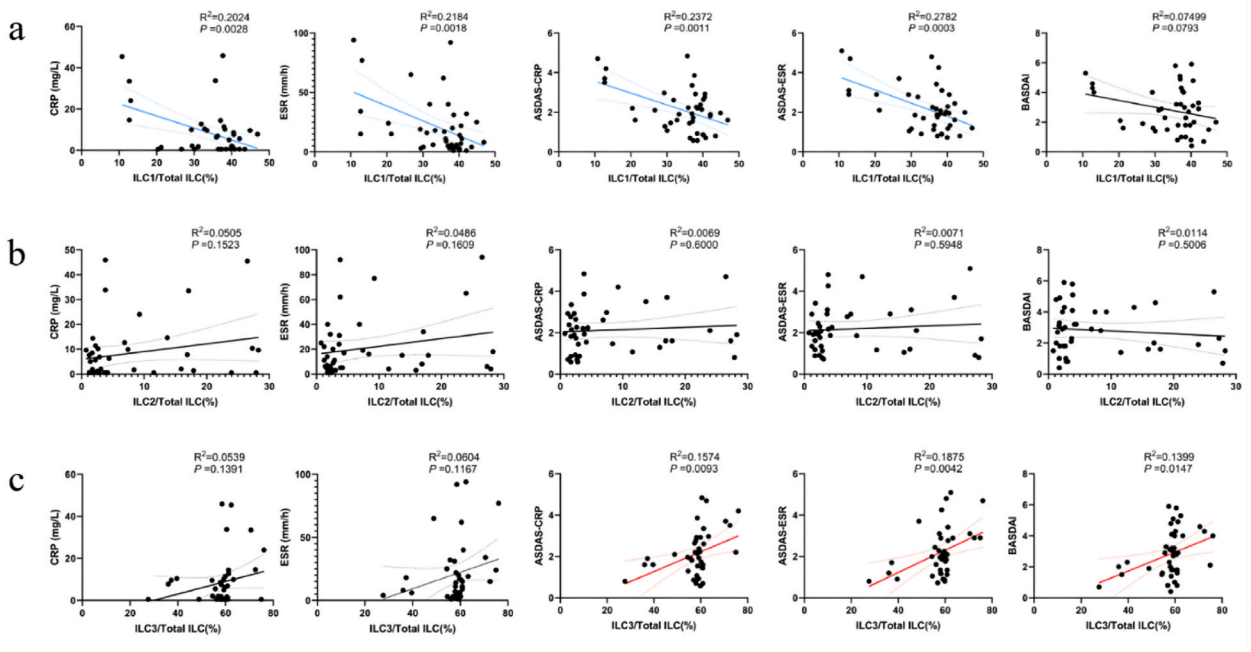

Fig. 4. Correlation analysis of each ILC subsets with inflammation level and disease activity in AS.
a. Correlation between the frequency of ILC1 in ILCs and inflammation level (CRP, ESR), disease activity score (ASDAS-CRP, ASDAS-ESR, BASDAI) (n = 42); b. Correlation between the frequency of ILC2 in ILCs and inflammation level (CRP, ESR), disease activity score (ASDAS-CRP, ASDAS-ESR, BASDAI); c. Correlation between the frequency of ILC3 in ILCs and inflammation level (CRP, ESR), disease activity score (ASDAS-CRP, ASDAS-ESR, BASDAI). Spearman Rank Correlation analysis was used to analyzed the correlation between ILCs and disease activity scores.

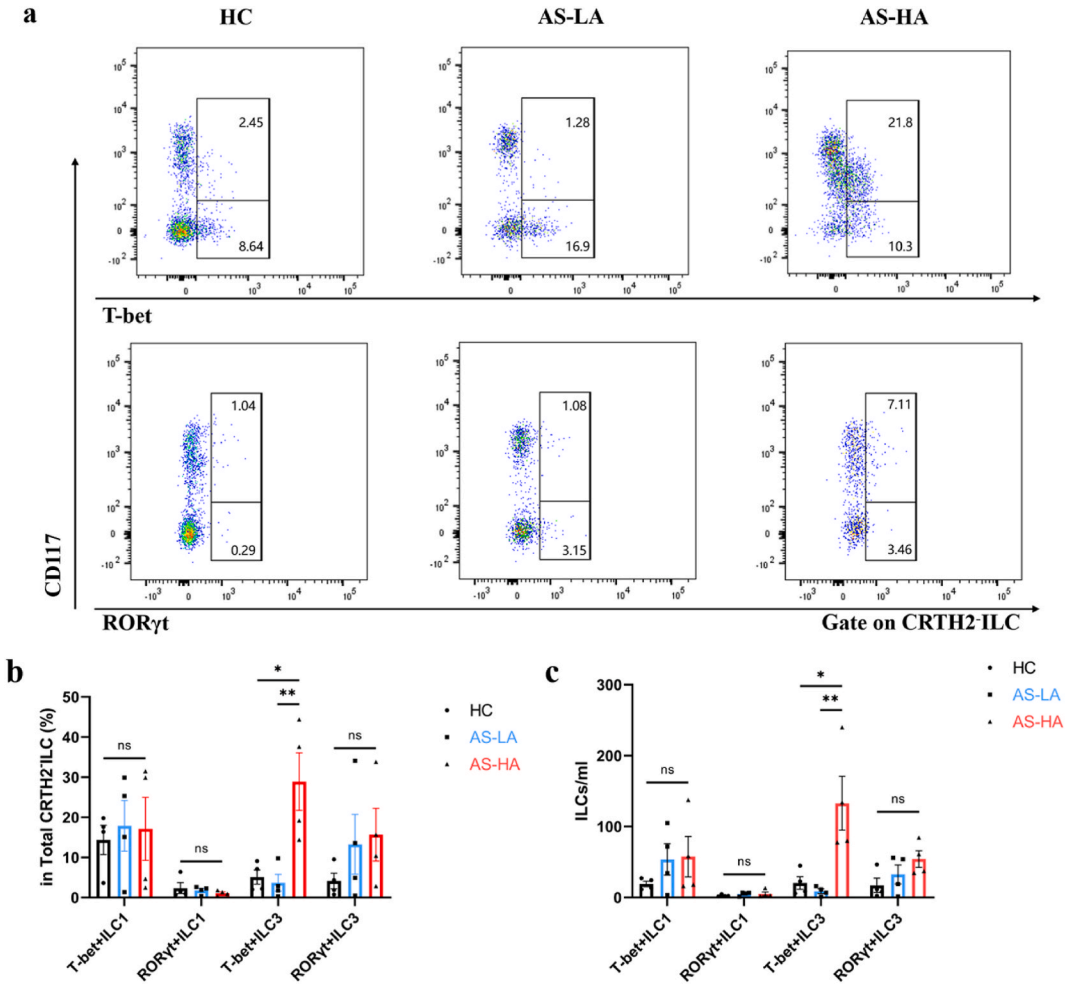

Fig. 5. Expression of transcription factors (T-bet, RORγt) in ILC subsets
a. Flow cytometry gating strategies for the identification on the expression of T-bet and RORγt in CRTH2$^-$ ILC; b. The percentages of T-bet$^+$ILC1, T-bet$^+$ILC3, RORγt$^+$ILC1 and RORγt$^+$ILC3 in CRTH2- ILC of the three groups (HC = 9, AS-LA = 5, AS-HA = 6); c. The absolute counts of T-bet$^+$ILC1, T-bet$^+$ILC3, RORγt$^+$ILC1 and RORγt$^+$ILC3 in the three groups(HC, AS-LA, AS-HA). Data are shown as means ± SD. Post-hoc pairwise comparisons were performed using the Least Significant Difference (LSD) test. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. *ns*, not significant.

To further determine the relationship between ILC3 distribution and the inflammation activity of AS, we analyzed 24 chemotactic and inflammatory cytokines in plasma samples from AS-LA and AS-HA groups using a Luminex liquid suspension chip. Patient characteristics are detailed in supple. Table 2. As shown in Fig. 6a and b, six cytokines- Eotaxin (also known as CCL11), IFN-γ, IL-17A, Monocyte chemoattractant protein-1 (MCP-1), Macrophage inflammatory protein (MIP)-1beta (MIP-1β) and Tumour necrosis factor alpha (TNF-α)- were significantly upregulated in the AS-HA group compared to the AS-LA group. Among these, the levels of IL-17A and IFN-γ correlated positively with the proportion of ILC3 in total ILCs, whereas the other cytokines, including Eotaxin, MCP-1, MIP-1β and TNF-α, did not show significant correlations (Fig. 6c). These results highlight a clear association between the distribution of ILC3 in the peripheral blood and the levels of IL-17A and IFN-γ cytokines. Previous studies have established that ILC3, like Th17 cells, is a major source of IL-17, while ILC1, akin to Th1 cells, primarily produces IFN-γ. Our results showed a marked amplification of T-bet$^+$ILC3 in AS patients with high disease activity (Fig. 4b and c). Based on this, we speculate that T-bet$^+$ILC3 may exhibit dual functionality, combining characteristics of both ILC1 and ILC3, and thereby contributing significantly to disease activity in AS. Further experimental validation is required to confirm this hypothesis. Nevertheless, our results strongly support the conclusion that the expansion of T-bet$^+$ILC3 is significantly correlated with disease activity in AS and closely linked to the increased release of IL-17A and IFN-γ.

4. Discussion

In this study, we provide compelling evidence that circulating ILCs expand in active AS, with a predominant increase in the ILC3 subset that correlates strongly with disease activity. We identified these expanded ILCs as Lin$^-$CD45$^+$CD127$^+$ cells, with the ILC3

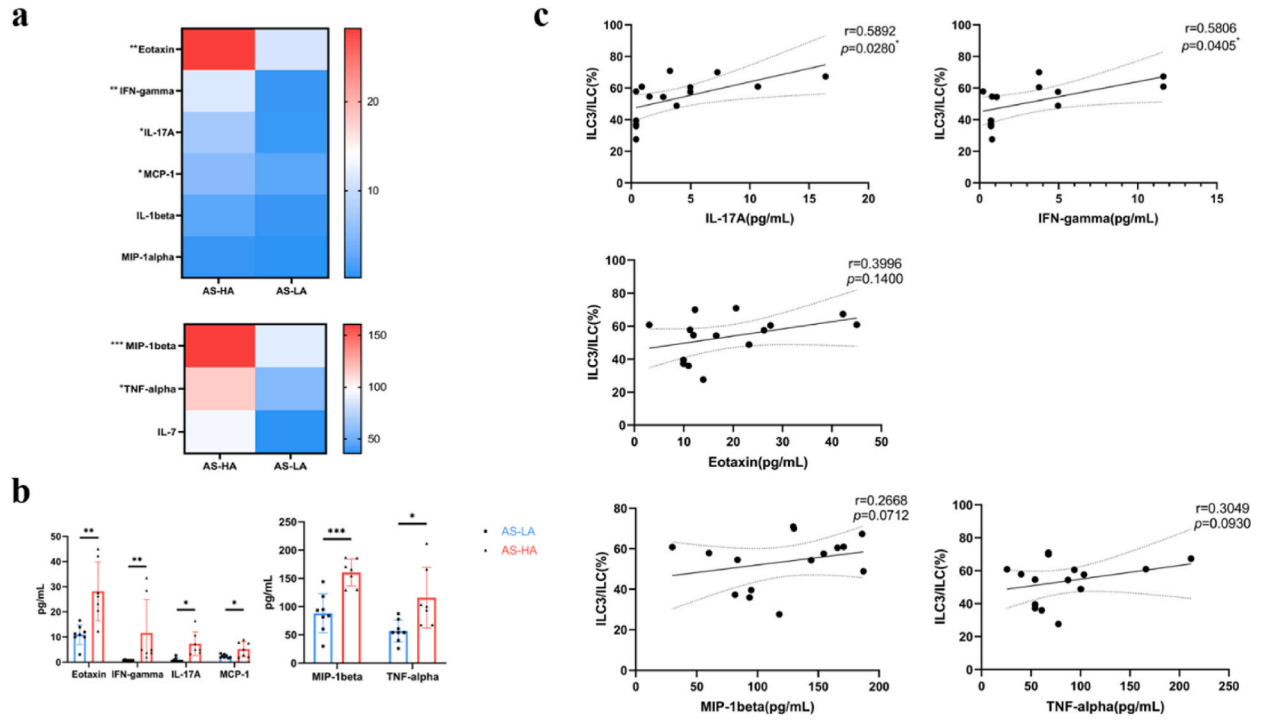

Fig. 6. Differential expression of cytokines by AS-HA and AS-LA

a.Luminex liquid suspension chip analysis of peripheral blood plasma from AS-HA(n = 7) and AS-LA(n = 8); b. Concentration comparison of differentially expressed cytokines between AS-LA and AS-HA; c. Correlation between the frequency of ILC3 in ILCs and Cytokine Levels (IL-17A, IFN-γ, Eotaxin, MCP-1, MIP-1β and TNF-α). Data are shown as means ± SD. Post-hoc pairwise comparisons were performed using the Least Significant Difference (LSD) test. Pearson correlation analysis was used to analyze the correlation between ILC3 and cytokines. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.*ns*, not significant.

subset specifically defined as CD117^{+}CRTH2^{-} cells. Interestingly, this subset of ILC3 predominantly expressed the transcription factor T-bet, indicating potential heterogeneity among ILC3 populations in the peripheral blood of AS patients. Results from Luminex liquid suspension chip analysis revealed increased levels of IL-17A and IFN-γ in the peripheral blood of AS-HA patients, correlating with the ILC3 population. Previous studies have shown that ILC3s are major producers of IL-17A, while ILC1s primarily secrete IFN-γ. However, our findings demonstrate a notable increase in the proportion of ILC3, particularly in T-bet^{+}ILC3, in AS patients with high disease activity. We hypothesize that T-bet^{+}ILC3 represents a novel subset of ILC3 in the peripheral blood of AS patients, possessing characteristics of both ILC1 and ILC3. This subset may play a critical role in regulating the inflammatory response and driving the pathogenesis of AS by facilitating the transition between ILC1 and ILC3. These results underscore its potential significance in the treatment of AS. Taken together, our findings suggest that peripheral circulating ILC3s, particularly T-bet^{+}ILC3, are actively involved in the pathogenesis of AS and exhibit unique heterogeneity.

ILC3s are a mucosal-restricted lymphoid cell population requiring transcription factors RORγt and T-bet for their differentiation. Unlike cytotoxic cells, ILC3s lack effector mechanisms such as perforin, granzymes and death receptors. A prior study demonstrated the expansion of T-bet^{+}ILC3s in the gut, peripheral blood, synovial fluid, and bone marrow of AS patients [13]. Consistent with these observations, our data also showed a significant increase in T-bet^{+}ILC3s in the peripheral blood in AS patients with high disease activity. Additionally, T-bet^{+}ILC3 were closely associated with inflammation and contributed to disease development in AS patients.

T-bet, initially identified in CD4^{+}T cells, is a key transcription factor defining the Th1 lineage. It plays a crucial role in regulating the expression of critical molecules like IFN-γ and CXCR3 and influences the functional differentiation of various immune cell subsets, including CD8^{+} T cells, NK cells, and B cells [20–22]. A study identified a genetic association between AS and rs11657479, a single nucleotide polymorphism (SNP) in the 3′ untranslated region (UTR) of TBX21, which encodes T-bet [23]. Homozygosity for the rs11657479 risk allele was associated with increased T-bet expression, particularly in CD8^{+} T cells, leading to elevated production of IFN-γ and IL-17 during disease progression [24]. Furthermore, TBX21 has been implicated in both AS and inflammatory bowel disease (IBD) [25]. T-bet is essential for the differentiation and maturation of both ILC1 and ILC3 [26]. In murine models of colitis, T-bet deletion reduced the capacity of ILC3 to produce IL-22 and IFN-γIn ILC [27]. Interestingly, sustained IL-23 stimulation in mice caused intestinal ILC3 to transition into a T-bet-expressing phenotype, suggesting the plasticity of ILCs [23,28]; ILC1 and ILC2 have been shown to convert into ILC3-like cells under inflammatory conditions [29,30]. Such transitions have been observed in psoriasis and IBD, where ILC2 transitioned to IL-17^{+}ILC3 and RORγt^{+}ILC3 converted into T-bet^{+}ILC1 [31–34]. These changes disrupt tissue homeostasis and contribute to disease pathogenesis. In AS, the mechanism underlying the transition from T-bet^{+}ILC1 to T-bet^{+}ILC3 or RORγt^{+}ILC3

to T-bet^{+}ILC3 remains unclear. Our results showed that significant amplification of T-bet^{+}ILC3s, which correlates closely with inflammation, particularly in IL-17A and IFN-γ level. This suggests that T-bet^{+}ILC3 may represent a distinct subset with dual functions, playing a role in maintaining inflammatory homeostasis in AS.

The association between SpA and intestinal inflammation is well established, with up to 50 % of patients of SpA patients suffer from subclinical or clinical intestinal inflammation [17]. Studies have linked ILC3 expansion to SpA-related intestinal inflammation. In enteropathic SpA, elevated levels of IL-17-producing ILC3 were observed in peripheral blood[36], whereas NKp44^{+}ILC3, which produces IL-22, predominated in the gut of AS patients [35]. α4β7^{+}ILC3, a gut-derived subset, was shown to migrate to α4β7 ligand-expressing joints, promoting local inflammation [13]. These findings suggest that ILC3s originating in the intestine may migrate via the peripheral circulation to extraintestinal sites, such as joints, where they mature and exert pro-inflammatory effects. However, the special roles of ILC3s in different tissue compartments remain to be elucidated. It is unclear whether the observed increase in T-bet^{+}ILC3s results from intestinal migration or from differentiation of ILC1 or ILC3 within peripheral blood. Further studies are required to clarify the functional dynamics of ILC3 in the intestine, peripheral circulation, and joints.

Our study demonstrates that T-bet^{+}ILC3 expansion is significantly associated with high disease activity in AS. Furthermore, T-bet^{+}ILC3 correlates strongly with the production of IL-17A and IFN-γ, cytokines implicated in sustaining chronic inflammation in AS. However, there are some limitations to our study. On the one hand, we did not directly detect the cytokines secreted by T-bet^{+}ILC3, and the precise biological functions of this specific ILC subtype have yet to be determined. On the other hand, the transdifferentiation between ILC1 and ILC3 cannot be fully determined solely from the perspective of cytokines and transcription factors; more detailed research is needed for further evaluation. However, our study at least confirms the correlation between T-bet^{+}ILC3 and disease activity in AS, suggesting its important value in the treatment of AS. These results expand our understanding of the pathoetiology of AS and inform potential therapies for mitigating the devastating impacts of this disease.

5. Conclusion

Our results suggest that T-bet^{+}ILC3s plays a key role in the inflammation development of AS disease and could be as a new therapy target for AS disease.

CRediT authorship contribution statement

Yang Liu: Writing – original draft, Formal analysis, Data curation. **Yi Shen:** Writing – original draft, Methodology, Data curation. **Hongbai Ding:** Formal analysis, Data curation. **Dongyi He:** Validation, Supervision. **Peng Cheng:** Methodology, Data curation. **Xinyao Wu:** Data curation. **Zheng Xiang:** Software, Data curation. **Lei Shen:** Writing – review & editing, Visualization, Validation. **Yanqin Bian:** Writing – review & editing, Visualization, Funding acquisition. **Qi Zhu:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

Ethics statement

This study was conducted in accordance with the ethical standards set forth in the Declaration of Helsinki and followed all applicable institutional and national guidelines for ethical research. Prior to initiating the research, the study protocol was reviewed and approved by the Ethics Committee of Shanghai University of Traditional Chinese Medicine (Ethics Approval No. 2020-K-45).

For studies involving human participants, informed consent was obtained from all subjects prior to their participation in the research. Participants were fully informed of the study's purpose, procedures, potential risks, and benefits, and their participation was entirely voluntary. Measures were taken to ensure confidentiality and privacy of the participants' data.

No identifiable personal data or images of study participants are included in this manuscript. In the case of datasets involving publicly available human data, ethical permissions were obtained from the respective data repositories.

The authors declare compliance with the Heliyon journal's ethical standards and confirm that all necessary approvals were obtained before the research commenced.

Consent for publication

The data presented in this manuscript have not been submitted or published elsewhere. All authors agree to the publication of this manuscript.

Availability of data and materials

The datasets used and/or analyzed during this study are available from the corresponding author upon reasonable request.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Yanqin Bian reports financial support was provided by National Natural Science Foundation of China. Qi Zhu reports financial support was provided by Shanghai Municipal Natural Science Foundation. Qi Zhu reports financial support was provided by Scientific research project of Shanghai Municipal Health Commission. Yanqin Bian reports financial support was provided by Training Program for High-caliber Talents of Clinical Research at Affiliated Hospital of Shanghai University of Traditional Chiese Medicie. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

Innate lymphoid cells ILCs
ankylosing spondylitis AS
C-reactive protein CRP
Erythrocyte sedimentation rate ESR
Ankylosing spondylitis disease activity score ASDAS
Bath ankylosing spondylitis disease activity index BASDAI
T-box transcription factor 21 T-bet;
Retinoid-related orphan receptor-gammat RORγt
AS patients with high disease activity AS-HA
Spondyloarthritis axSpA
(axSpA); Spondyloarthritis SpA
interleukin-17A IL-17A
Type 1 ILCs ILC1s
Type 2 ILCs ILC2s
Type 3 ILCs ILC3s
Peripheral blood mononuclear cells PBMCs
lineage-negative, Lin
Monocyte chemoattractant protein-1 MCP-1
Macrophage inflammatory protein MIP
Tumour necrosis factor alpha TNF-α.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2025.e41678.

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