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Original Article

Expanded IL-22$^+$ Group 3 Innate Lymphoid Cells and Role of Oxidized LDL-C in the Pathogenesis of Axial Spondyloarthritis with Dyslipidaemia

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ABSTRACT

Group 3 innate lymphoid cells (ILC3), which express IL-22 and IL-17A, has been introduced as one of pathologic cells in axial spondyloarthritis (axSpA). Dyslipidaemia should be managed in axSpA patients to reduce cardiovascular disease, and dyslipidaemia promotes inflammation. This study aimed to reveal the role of circulating ILC3 in axSpA and the impact of dyslipidaemia on axSpA pathogenesis. AxSpA patients with or without dyslipidaemia and healthy control were recruited. Peripheral blood samples were collected, and flow cytometry analysis of circulating ILC3 and CD4$^+$ T cells was performed. The correlation between Ankylosing Spondylitis Disease Activity Score (ASDAS)-C-reactive protein (CRP) and circulating immune cells was evaluated. The effect of oxidized low-density lipoprotein cholesterol (oxLDL-C) on immune cell differentiation was confirmed. AxSpA human monocytes were cultured with with oxLDL-C, IL-22, or oxLDL-C plus IL-22 to evaluate osteoclastogenesis using tartrate-resistant acid phosphatase (TRAP) staining and real-time quantitative PCR of osteoclast-related gene expression. Total of 34 axSpA patients (13 with dyslipidaemia and 21 without) were included in the analysis. Circulating IL-22$^+$ ILC3 and Th17 were significantly elevated in axSpA patients with dyslipidaemia (p=0.001 and p=0.034, respectively), and circulating IL-22$^+$ ILC3 significantly correlated with ASDAS-CRP (Rho=0.4198 and p=0.0367). Stimulation with oxLDL-C significantly increased IL-22$^+$ ILC3, NKp44$^-$ ILC3, and Th17 cells, and these were reversed by CD36 blocking agent. IL-22 and oxLDL-C increased TRAP$^+$ cells and osteoclast-related gene expression. This study suggested potential role of circulating IL-22$^+$ ILC3 as biomarker in axSpA. Furthermore, dyslipidaemia augmented IL-22$^+$ ILC3 differentiation, and oxLDL-C and IL-22 markedly increased osteoclastogenesis of axSpA.

Keywords: Spondyloarthritis; Group 3 innate lymphoid cells; Interleukin-22; Dyslipidemias; Osteoclasts

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Conflict of Interest
The authors declare no potential conflicts of interest.

Abbreviations
axSpA, axial spondyloarthritis; ASDAS, ankylosing spondylitis disease activity score; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; BMI, body mass index; BV, Brilliant Violet; CRP, C-reactive protein; CTR, calcitonin receptor; Cy, cyanine; DM, diabetes mellitus; ESR, erythrocyte sedimentation rate; HTN, hypertension; ILC, innate lymphoid cell; LDL-C, low-density lipoprotein cholesterol; ns, not significant; NSAID, non-steroidal anti-inflammatory drug; oxLDL-C, oxidized low-density lipoprotein cholesterol; PGA, patient's global assessment; RA, rheumatoid arthritis; RANKL, receptor activator of nuclear factor kappa B ligand; RT-qPCR, real-time quantitative polymerase chain reaction; SpA, spondyloarthritis; TRAP, tartrate-resistant acid phosphatase.

Author Contributions
Conceptualization: Min HK, Cho ML, Park SH; Data curation: Min HK, Moon J, Lee SY, Lee J, Kwok SK; Formal analysis: Lee SY, Lee AR; Funding acquisition: Park SH; Investigation: Min HK, Moon J, Lee CR, Cho ML; Methodology: Lee SY, Cho ML; Supervision: Lee J, Kwok SK, Cho ML, Park SH; Validation: Min HK, Kwok SK, Park SH; Visualization: Min HK; Writing - original draft: Min HK; Writing - review & editing: Cho ML, Park SH.

INTRODUCTION

Spondyloarthritis (SpA) is a systemic inflammatory arthritis that can be divided into axial SpA (axSpA) and peripheral SpA (1). AxSpA not only causes articular symptoms but also irreversible bony ankyloses of the axial joints, which eventually cause "bamboo spine." The main goals of SpA treatment are suppressing the inflammatory response to reduce articular symptoms and preventing irreversible bony ankyloses. Current treatment guidelines for axSpA recommend the use of non-steroidal anti-inflammatory drugs and biologic agents (2). However, some patients still do not respond to currently available medications, and the development of new treatment modalities is required.

The IL-23 and IL-17 axes are the main pathological processes of axSpA (3). In fact, IL-17A blocking agents, such as secukinumab and ixekizumab, have shown therapeutic effects on SpA (2). However, the IL-23/IL-12 p40 blocking agent ustekinumab failed to exert significant therapeutic effects against axSpA (4). The discordant results between the theoretical background and actual efficacy in patients with axSpA have resulted in the need to identify the major pathologic cells of axSpA. Specifically targeting these crucial pathologic immune cells may increase the therapeutic efficacy.

Innate lymphoid cells (ILCs) are a unique subset of immune cells in the innate immune system (5). ILCs can be divided into 5 types: 1) NK cells, 2) group 1 ILC (ILC1), 3) ILC2, 4) ILC3, and 5) lymphoid tissue-inducer cells (6). ILCs are abundant in the barrier surface of the body, including the gastrointestinal tract. Recently, several studies have demonstrated a link between ILCs and the pathogenesis of inflammatory arthritis (7). With respect to axSpA, ILC3 was upregulated in the peripheral blood, bone marrow, synovial fluid, and gut tissue of axSpA patients (8). Furthermore, IL-22-expressing ILC3 was upregulated in the inflamed joints of patients with SpA (9). Based on these previous results, ILCs have been suggested as "cytokine shuttle" immune cells that travel from the gastrointestinal tract to the joints (10).

Spinal structural progression in axSpA is irreversible and reduces the motility of the axial joints. These changes occur in several steps: 1) acute inflammation, 2) erosion, 3) enchondral ossification, and 4) abnormal new bone formation at the spinal corner (11). Erosive changes precede abnormal new bone formation, also called syndesmophytes, and erosion on the vertebral corner is primarily mediated by osteoclasts. Osteoporosis is the most frequent co-morbidity in axSpA and can occur even in the early stages of axSpA (12,13). This supports the hypothesis that osteoclast activity is upregulated in axSpA. Furthermore, inflammatory lesions in spine magnetic resonance images previously revealed osteoclast dominance in axSpA (14). Osteoclastogenesis is promoted by receptor activator of nuclear factor kappa B ligand (RANKL)-RANK interactions; however, several cytokines can also increase osteoclastogenesis. Among them, IL-22 is known to promote osteoclastogenesis in rheumatoid arthritis (RA) (15). However, the role of IL-22 in the osteoclastogenesis of axSpA has not yet been elucidated.

The relationship between dyslipidaemia and inflammation has been identified. In RA patients with a high inflammatory burden, lipid levels were initially low and then increased after disease activity was controlled (16). The negative associations between inflammatory status and lipid levels are evident, but the underlying mechanism has not been fully elucidated. In addition, low-density lipoprotein cholesterol (LDL-C) may affect the development of immune cells and the inflammatory burden. LDL-C induces an inflammatory

response of atherosclerosis by promoting toll-like receptor signalling in macrophages (17). In addition, LDL-C induces epigenetic changes in hematopoietic stem and progenitor cells, which promote the proliferation and mobilisation of neutrophils and monocytes (18). Oxidized LDL-C (oxLDL-C) stimulation has been observed to change ILC populations (19). Furthermore, oxLDL-C promoted osteoclastogenesis was observed in several animal models (20,21). These results suggest that dyslipidaemia is associated with the inflammatory response and bone homeostasis.

In this study, we evaluated the percentage of circulating ILC3 and $CD4^+$ T cells (IFN-γ^+ $CD4^+$ T cells, IL-$17A^+$ $CD4^+$ T cells, and IL-22^+ $CD4^+$ T cells) in axSpA patients and examined the correlation between these immune cells and disease activity. Furthermore, we hypothesised that axSpA patients with dyslipidaemia may have increased pathologic immune cells in their peripheral blood and compared these levels between axSpA patients with and without dyslipidaemia. In addition, the role of oxLDL-C in ILC3 differentiation and those of ILC3-expressing cytokines and oxLDL-C in axSpA osteoclastogenesis were evaluated.

MATERIALS AND METHODS

Patients

Patients with axSpA were recruited from 2 tertiary hospitals, Konkuk University Medical Center (Seoul, Korea) and Seoul St. Mary's Hospital (Seoul, Korea). The inclusion criteria were as follows: 1) fulfilment of either the 1984 modified New York criteria for ankylosing spondylitis or the 2009 Assessment of SpondyloArthritis International Society classification criteria for axSpA, and 2) age >19 years old. The exclusion criteria were as follows: (1) presence of another autoimmune disease or inflammatory arthritis, (2) presence of infection, or (3) presence of malignancy. Peripheral blood was obtained from patients with axSpA and healthy controls after obtaining informed consent from all patients. Disease activity parameters and the Ankylosing Spondylitis Disease Activity Score (ASDAS)-C-reactive protein (CRP) were evaluated (22). Patients whose LDL-C levels were higher than 130 mg/dL were defined as having dyslipidaemia (23). The experimental protocol was approved by the Institutional Review Boards of Konkuk University Medical Center (KUH 2019-08-015) and Seoul St. Mary's Hospital (KC18TESI0627).

Isolation and stimulation of PBMCs

Human PBMCs were collected from heparinised blood by standard Ficoll-Paque density gradient centrifugation (GE Healthcare Biosciences, Uppsala, Sweden). Isolated PBMCs were cultured in Roswell Park Memorial Institute-1640 medium (Gibco BRL, Carlsbad, CA, USA) containing penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% foetal bovine serum (Gibco BRL), which was previously inactivated by heating to 55°C for 30 min. PBMCs were seeded into 48-well plates (Nunc, Rosklide, Denmark). To investigate the population of $CD4^+$ T cells expressing IFN-γ, IL-17A, and IL-22 as well as ILC3, the cells were stimulated with phorbol myristate acetate and ionomycin with GolgiStop for 4 h (BD Biosciences, San Jose, CA, USA). For analysis of the oxLDL-C effect, PBMCs were cultured under oxLDL-C stimulation (L34357, 50 μg/ml; Thermo Fisher, Waltham, MA, USA) for 72 h. To determine, whether impact of oxLDL-C on differentiation of $CD4^+$ T cells and ILC3 cells were mediated by oxLDL-C receptor, CD36 (24), we also used CD36 blocking Ab (MA5-14112; Invitrogen, Carlsbad, CA, USA).

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Flow cytometry analysis

To quantify IFN-γ-, IL-17A-, and IL-22-positive cells in $CD4^+$ T cells, PBMCs were immunostained using a PE-cyanine (Cy)7-conjugated anti-CD4 Ab (300512; BioLegend, San Diego, CA, USA), fixed, and permeabilised using a Cytofix/Cytoperm Plus kit (BD Biosciences). Following the manufacturer's instructions, PBMCs were stained with fluorescein isothiocyanate-conjugated anti-IL-17A (11-7179-42; eBiosciences, San Diego, CA, USA), PE-conjugated anti-IL-22 (12-7229-42; eBiosciences), and allophycocyanin-conjugated anti-IFN-γ (506510; BD Biosciences) Abs.

To identify ILC3, PBMCs were surface-labelled with peridinin-chlorophyll-protein Cy5.5-conjugated anti-CD45 (564105; BD Biosciences), Brilliant Violet (BV)510-conjugated anti-CD3 (564713; BD Biosciences), PE-Cy7-conjugated anti-CD127 (560822; BD Biosciences), Brilliant Blue 515-conjugated anti-CD117 (565172; BD Biosciences), Alexa Fluor 647-conjugated anti-NKp44 (558564; BD Biosciences), PE-conjugated anti-CD294 (563665; BD Biosciences), and PE-conjugated anti-β7 integrin (321203; Biolegend) Abs, followed by fixation, permeabilisation, and intracellular staining with BV421-conjugated anti-IL-17A (562933; BD Biosciences) and PE-conjugated anti-IL-22 (eBiosciences) Abs. To identify oxLDL receptor, CD36, on ILC3, we used peridinin-chlorophyll-protein Cy5.5-conjugated anti-CD36 (336224; Biolegend). All cells were detected using a FACSCalibur device (BD Pharmingen, Franklin Lakes, NJ, USA).

ELISA of TNF-α, IL-17A, and IL-22 measurement in serum

Briefly, a 96-well plate (Eppendorf, Hamburg, Germany) was coated with monoclonal Abs against TNF-α (DY210; R&D systems, Minneapolis, MN, USA), IL-17A (DY317; R&D systems), and IL-22 (MAB7822, 782-IL, BAM7821; R&D systems) at 4°C overnight. After blocking with PBS/1% bovine serum albumin/0.05% Tween 20 for 2 h at room temperature (22°C–25°C), the test samples and recombinant TNF-α, IL-17A, and IL-22 as standards were added to the 96-well plate and incubated at room temperature for another 2 h. The plates were washed 4 times with PBS and Tween 20, and then incubated with biotinylated human monoclonal Abs against TNF-α, IL-17A, and IL-22 for 2 h at room temperature. After washing, the streptavidin–alkaline phosphate–horseradish peroxidase conjugate (Sigma, St. Louis, MA, USA) was added to the wells for 30 min, and incubation with 1 mg/ml *p*-nitrophenyl phosphate (Sigma) dissolved in diethanolamine (Sigma) to develop the color reaction. The reaction was stopped by the addition of 1 M NaOH, and the optical density of each well was measured at 405 nm.

In vitro osteoclastogenesis and tartrate-resistant acid phosphatase (TRAP) staining

$CD14^+$ monocytes (1×10^6 cells) from human PBMCs were plated in 24-well (Nunc) and cultured in alpha-minimal essential medium (Invitrogen) containing antibiotics and 10% heat-inactivated foetal bovine serum to separate floating and adherent cells. Nonadherent cells were removed by washing with PBS, and preosteoclasts were cultured in the presence of 25 ng/ml M-CSF and 30 ng/ml RANKL (PeptoTech, London, UK). To investigate the impact of oxLDL-C and IL-22 on osteoclast differentiation, 10 μg/ml oxLDL-C, 10 or 50 ng/ml IL-22, and 10 μg/ml oxLDL-C plus 10 or 50 ng/ml IL-22 were added to culture media. Additionally, to investigate the impact of IL-22 blocking agent on oxLDL-C and IL-22 induced osteoclastogenesis, 10 μg/ml oxLDL-C + 50 ng/ml IL-22, and 10 μg/ml oxLDL-C + 50 ng/ml IL-22 + 2 μg/ml IL-22 blocking Ab (16-7222-82; eBiosciences) added condition of *in vitro* osteoclast differentiation experiments were performed. The medium was changed every

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3 days. Osteoclasts were generated after 14 days. A commercial TRAP kit (Sigma-Aldrich, St. Louis, MO, USA) was used according to the manufacturer's instructions; however, counterstaining with haematoxylin was not performed. TRAP-positive multinuclear cells containing 3 or more nuclei were counted as osteoclasts. Three independent person counted multinucleated TRAP-positive cells, and experiment conditions were blinded.

Expression levels of osteoclast-associated genes by real-time quantitative PCR (RT-qPCR) analysis

Cathepsin K, *TRAP*, *calcitonin receptor* (*CTR*), *RANK*, *c-Fos*, *OSCAR*, and *NFATc1* mRNA expression levels were determined by RT-qPCR analysis with SYBR Green I (Roche Diagnostics, Mannheim, Germany). Reaction mixtures were amplified in a LightCycler (Roche Diagnostics). Fluorescence curves were analysed using LightCycler software v.3.0 (Roche Diagnostics). Expression levels were calculated and normalised to the value of the housekeeping gene (beta-actin).

Statistical analysis

Continuous variables were assessed by the Kolmogorov-Smirnov test for normality and then analysed using the Student's *t*-test or the Mann-Whitney *U* test. The data are expressed as means±standard deviations or medians with interquartile ranges. Spearman's correlation test was used to determine the correlation between disease activity and circulating immune cells. In all analyses, $p<0.05$ indicated statistical significance.

RESULTS

Characteristics of enrolled axSpA patients

A total of 34 axSpA patients comprising 13 with dyslipidaemia and 21 without dyslipidaemia, and 7 healthy controls were included. The median age of axSpA patients with dyslipidaemia was 48 years, whereas that of axSpA patients without dyslipidaemia was 38. The median body mass index was significantly higher in axSpA patients with dyslipidaemia than in those without dyslipidaemia (24.7 vs. 23.0 kg/m^2, respectively). Other detailed information is summarised in **Table 1**.

Circulating ILC3 and CD4$^+$ T cells in axSpA and correlations with disease activity

PBMCs were analysed by flow cytometry to assess the populations of ILC3 and CD4$^+$ T cells in axSpA patients with and without dyslipidaemia. Gating strategies for CD4$^+$ T cells (IFN-γ$^+$ CD4$^+$ T cells, IL-17A$^+$ CD4$^+$ T cells, and IL-22$^+$ CD4$^+$ T cells), IL-17A$^+$ NKp44$^-$ ILC3, and IL-22$^+$ ILC3 are presented in **Supplementary Fig. 1A-C**. **Supplementary Fig. 1D and E** demonstrate flow cytometry image of unstained control of IL-17A$^+$ ILC3 and IL-22$^+$ ILC3. The percentage of each CD4$^+$ T cell type was calculated among the total lymphocyte population. The percentages of IL-17A$^+$ NKp44$^-$ and IL-22$^+$ ILC3 were calculated among the total ILC3 population (CD3$^-$ CD127$^+$ CD117$^+$ CD294$^-$). The frequencies of total ILCs and ILC3 among total PBMCs in axSpA patients with and without dyslipidaemia were 3.84±1.36% vs. 4.78±5.98% (p=0.56) and 0.69±0.68% vs. 0.44±0.34% (p=0.30), respectively. The total IL-17A$^+$ CD4$^+$ T cells (2.64±3.60%) and IL-22$^+$ CD4$^+$ T cells (1.79±1.22%) were significantly higher than total ILC3 (0.54±0.50%) in peripheral blood ($p<0.001$ and p=0.003, respectively). The frequencies of IL-22$^+$ ILC3 and IL-17A$^+$ CD4$^+$ T cells were significantly higher in axSpA patients with dyslipidaemia than in those without dyslipidaemia (30.07±11.94% vs. 10.71±6.18%, p=0.001 and 2.36±1.29% vs. 1.45±0.78%,

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Table 1. Information of demographics, laboratory, and disease related parameters

Characteristic	Healthy control (n=7)	Total axSpA (n=34)	AxSpA with dyslipidaemia (n=13)	AxSpA without dyslipidaemia (n=21)	p*
Sex (male)	6 (85.7)	30 (88.2)	12 (92.3)	18 (85.7)	0.974
Age (yr)	40.5 (35.0–46.0)	41.5 (36.0–48.0)	48.0 (41.0–50.0)	38.0 (30.0–46.0)	0.073
Disease duration (mon)		119.0 (61.5–179.0)	112.0 (78.0–201.0)	123.0 (47.0–179.0)	0.606
BMI (kg/m^2)	24.0 (22.1–27.0)	23.4 (21.3–26.0)	24.7 (23.3–28.7)	23.0 (19.9–24.2)	0.020
Ankylosing Spondylitis		20 (58.8)	9 (69.2)	11 (52.4)	0.541
HTN		4 (11.8)	3 (23.1)	1 (4.8)	0.288
DM		0	0	0	
Dyslipidaemia		13 (38.2)			
ESR (mm/hr)		5.0 (2.0–8.5)	5.0 (2.0–7.0)	5.0 (2.0–12.0)	0.514
CRP (mg/dL)		0.1 (0.0–0.2)	0.1 (0.0–0.1)	0.1 (0.0–0.2)	0.362
HLA-B27 positivity		33 (97.1)	13 (100.0)	20 (95.2)	1.000
TNF-a inhibitor user		26 (76.5)	10 (76.9)	16 (76.2)	1.000
NSAID user		24 (70.6)	10 (76.9)	14 (66.7)	0.802
PGA (0–10)		3.0 (2.0–5.0)	3.0 (2.0–4.0)	3.0 (1.5–5.0)	0.801
BASDAI		2.3 (1.1–4.8)	2.4 (2.1–4.0)	2.2 (1.1–5.2)	0.470
ASDAS-CRP		1.7 (1.1–2.5)	1.7 (1.3–2.1)	1.6 (1.1–2.5)	1.000
ASDAS-ESR		1.8 (0.9–2.4)	1.8 (1.2–2.0)	1.6 (0.9–2.5)	0.901

Values are presented as number (%) or median (range).
BMI, body mass index; HTN, hypertension; DM, diabetes mellitus; ESR, erythrocyte sedimentation rate; NSAID, non-steroidal anti-inflammatory drug; PGA, patient's global assessment.
*The p values for comparison between axSpA with and without dyslipidaemia groups.

p=0.034, respectively; **Fig. 1A**). The frequency of IL-17A$^+$ CD4$^+$ T cell were comparable between axSpA patients without dyslipidaemia and healthy control, whereas IL-22$^+$ ILC3 frequency were significantly higher in axSpA without dyslipidaemia than healthy control (**Fig. 1A**). The gut homing receptor of ILC3, α4β7 integrin (25), was dominantly expressed in NKp44$^-$ ILC3 (**Fig. 1B**). The frequency of IL-22$^+$ ILC3 was significantly positively correlated with ASDAS-CRP (Rho=0.4198 and p=0.0367; **Fig. 2**).
Neither IL-17A$^+$ NKp44$^-$ ILC3 nor IL-17A$^+$ CD4$^+$ T cells correlated with ASDAS-CRP. The correlation between ASDAS-CRP and frequency of IL-22$^+$ ILC3 were insignificant when dividing axSpA patients into with or without dyslipidaemia (**Supplementary Fig. 2**). The serum levels of IL-22, IL-17A, and TNF-α did not demonstrate significant correlation with ASDAS-CRP (**Supplementary Fig. 3**).

Impact of oxLDL-C on ILC3 and CD4$^+$ T cell differentiation

To investigate whether dyslipidaemia, increased LDL-C levels, could affect the proliferation of ILC3 cells, PBMCs from axSpA patients (n=10) were cultured under stimulation with oxLDL-C. The proportion of IL-22$^+$ ILC3 significantly increased under 50 μg/ml oxLDL-C stimulation relative to the nil condition (**Fig. 3A**). IL-17A$^+$ NKp44$^-$ ILC3 and IL-17A$^+$ CD4$^+$ T cells were also increased in the 50 μg/ml oxLDL-C stimulation condition compared to the nil condition (**Fig. 3A**). To discriminate either oxLDL-C directly influence or indirectly influence on ILC3 differentiation, we examined oxLDL-C receptor, CD36, on ILC3. CD36 was expressed in ILC3 population (**Fig. 3B**). Furthermore, to identify whether these were mediated by oxLDL-C receptor, CD36, we used CD36 blocking Ab (2 μg/ml). Co-administration of CD36 blocking Ab attenuated oxLDL-C induced differentiation of IL-22$^+$ ILC3 and IL-17A$^+$ CD4$^+$ T cells (**Fig. 3A**). These results suggest a potential pathological role of oxLDL-C in axSpA pathogenesis by inducing pathologic immune cells (IL-22$^+$ ILC3, IL-17A$^+$ NKp44$^-$ ILC3, and IL-17A$^+$ CD4$^+$ T cells), and these process is induced by CD36 mediated signal pathway.

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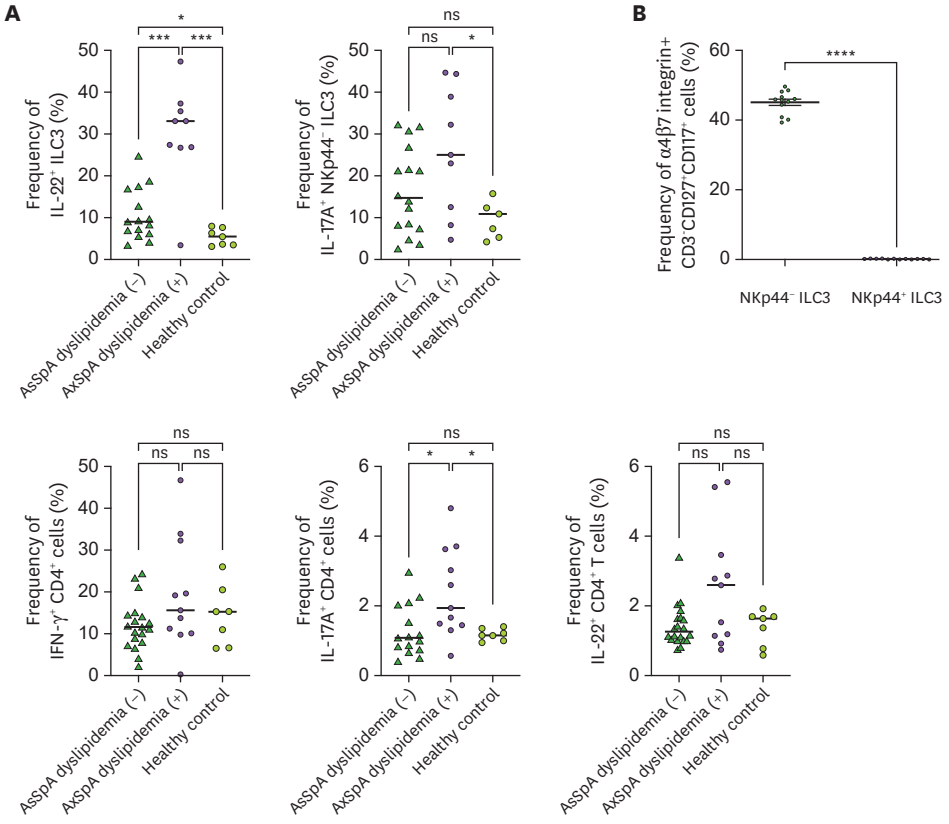

Figure 1. Circulating ILC3 and CD4⁺ T cells in axSpA patients with and without dyslipidaemia and healthy control. The PBMCs of axSpA patients were pre-stimulated with phorbol myristate acetate and ionomycin with GolgiStop for 4 h, then stained with T helper cells and ILC3-specific Abs. (A) Comparison of peripheral blood IL-22⁺ ILC3, IL-17A⁺ NKp44⁻ ILC3, IFN-γ⁺ CD4⁺ cells, IL-17A⁺ CD4⁺ cells, and IL-22⁺ CD4⁺ cells between axSpA patients with and without dyslipidaemia and healthy control. (B) Expression of α4β7 integrin on NKp44⁺ and NKp44⁻ ILC3.
ns, not significant.
*p<0.05; ***p<0.001; ****p<0.0001.

Effect of oxLDL-C and IL-22 on osteoclastogenesis

Human CD14⁺ monocytes from axSpA patients (n=5) were cultured under stimulation with oxLDL-C (10 μg/ml), IL-22 (10, 50 ng/ml), or oxLDL-C (10 μg/ml) plus IL-22 (10, 50 ng/ml) with M-CSF (25 ng/ml) and RANKL (30 ng/ml). Co-stimulation with IL-22 10 and 50 ng/ml plus oxLDL-C 10 μg/ml increased TRAP⁺ cell count compared to the M-CSF plus RANKL condition (**Fig. 4A and B**).

The expression levels of osteoclast-related genes *CTR*, *OSCAR*, *NFATc1*, *TRAP*, and *cathepsin K* were significantly higher in the IL-22 10 and 50 ng/ml plus oxLDL-C 10 μg/ml co-stimulation condition (**Fig. 4C**). The expression levels of *RANK* and *c-Fos* tend to be decreased in IL-22 10 ng/ml plus oxLDL-C 10 μg/ml condition, but the decrease was insignificant. To verify the effects of IL-22 on oxLDL-C induced osteoclastogenesis, we additionally performed *in vitro* osteoclast differentiation experiment by adding oxLDL-C (10 μg/ml) + IL-22 (50 ng/ml), or oxLDL-C (10 μg/ml) + IL-22 (50 ng/ml) + IL-22 blocking Ab (2 μg/ml) in M-CSF (25 ng/ml) and RANKL (30 ng/ml) condition. Stimulation with oxLDL-C 10 μg/ml + IL-22 50 ng/ml increased TRAP⁺ multinucleated cell count compared to the M-CSF plus RANKL condition, and TRAP⁺ osteoclast count was decreased by co-administration of IL-22 blocking Ab (**Supplementary Fig. 4A and B**). The expression levels of osteoclast-related genes, *CTR*, *c-Fos*, *OSCAR*, *NFATc1*, *TRAP*, and *cathepsin K*, were significantly increased in oxLDL-C 10 μg/ml + IL-22 50 ng/ml stimulation condition, whereas these were decreased by adding IL-22 blocking Ab (**Supplementary Fig. 4C**).

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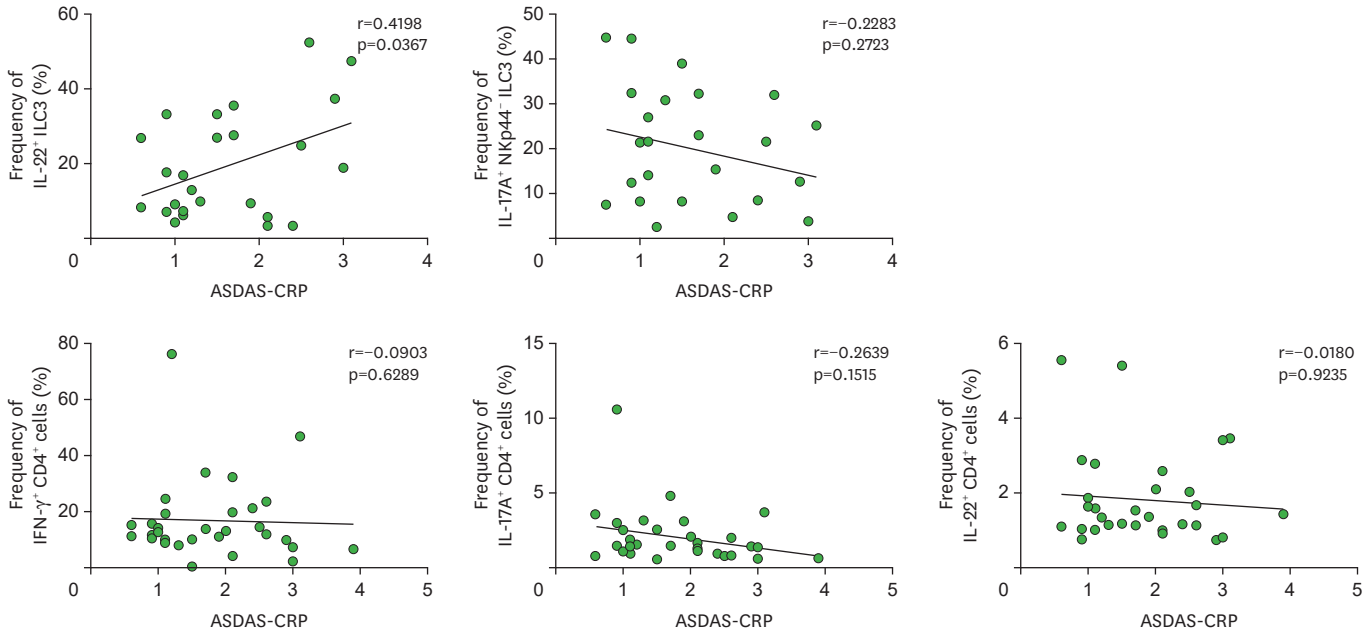

Figure 2. Correlation between circulating immune cells and ASDAS-CRP. ILC3 cells in patients with axSpA (n=34) were analysed by flow cytometry. Spearman correlation coefficients were calculated between circulating IL-22+ ILC3, IL-17A+ NKp44- ILC3, IFN-γ+ CD4+ cells, IL-17A+ CD4+ cells, IL-22+ CD4+ cells, and ASDAS-CRP.

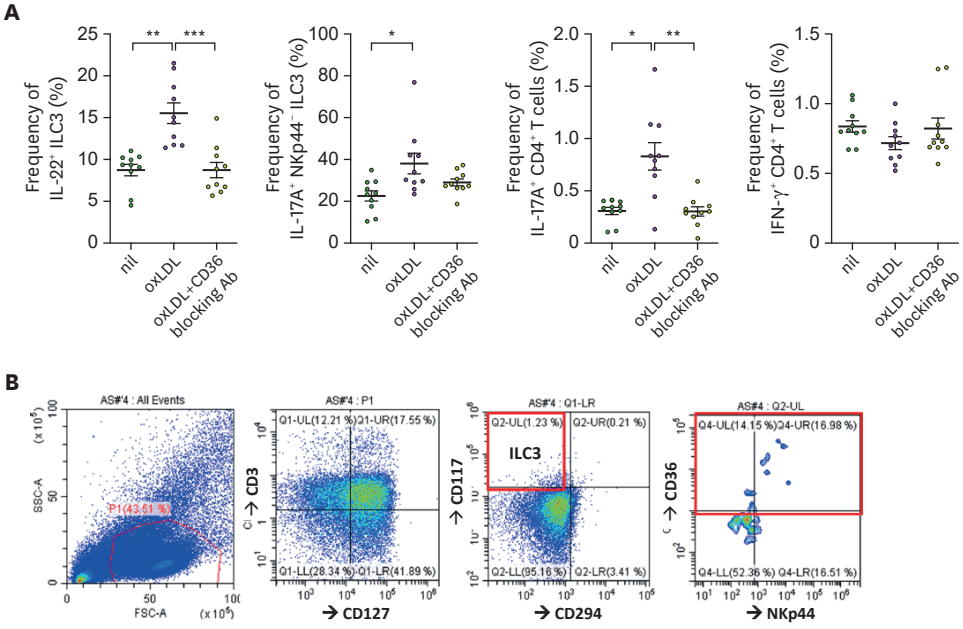

Figure 3. Effects of oxLDL-C on the differentiation of IL-22+ ILC3, IL-17A+ NKp44- ILC3, and IL-17A+ CD4+ cells. (A) *In vitro*, oxLDL-C (50 μg/ml) or oxLDL-C (50 μg/ml) + CD36 blocking Ab (2 μg/ml) was added to the PBMCs (5×10^5 cells/well) of axSpA patients (n=10) for 24 h. The frequencies of IL-22+ ILC3, IL-17A+ NKp44- ILC3, IL-17A+ CD4+, and IFN-γ+ CD4+ cells were measured by flow cytometry. (B) Flow cytometry gating strategy for CD36+ ILC3 (CD3- CD127+ CD117+ CD294- CD36+).
*p<0.05; **p<0.01; ***p<0.001.

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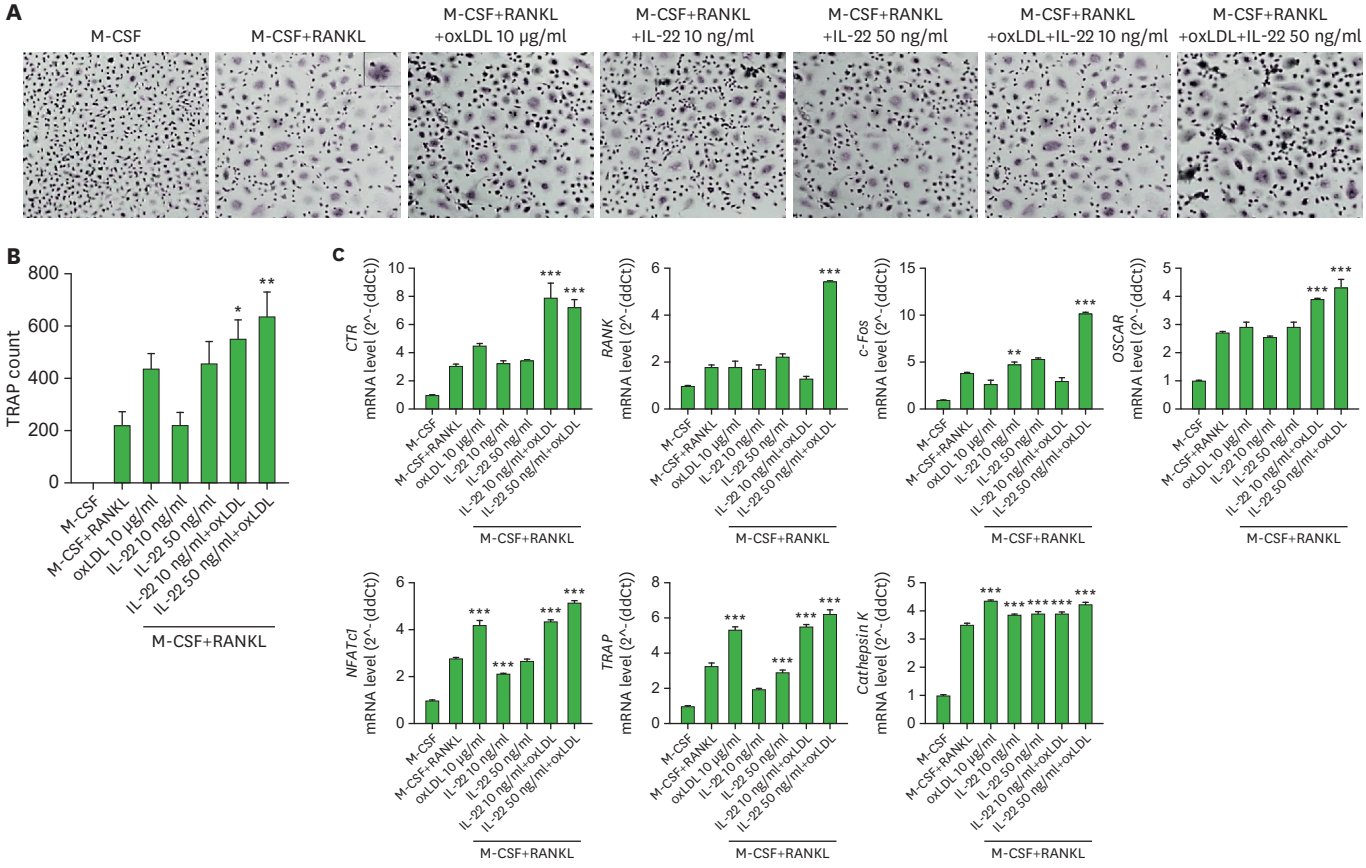

Figure 4. Effects of IL-22 and oxLDL-C on the osteoclastogenesis of axSpA-derived peripheral blood CD14⁺ monocytes. Peripheral blood CD14⁺ monocytes (1×10^6 cells) were obtained from axSpA patients (n=5). (A) These were cultured with oxLDL-C (10 μg/ml), IL-22 (10 and 50 ng/ml), or oxLDL-C (10 μg/ml) plus IL-22 (10 and 50 ng/ml) containing M-CSF (25 ng/ml) and RANKL (30 ng/ml) and stained for TRAP. Experiment conditions were blinded, and mean TRAP⁺ cells number counted by 3 individuals were used. (B) TRAP⁺ multinucleated cells were counted. (C) *CTR*, *RANK*, *c-Fos*, *OSCAR*, *NFATc1*, *TRAP*, and *Cathepsin K* mRNA expression levels were measured by real-time quantitative polymerase chain reaction. Data were normalised to beta-actin and reported in relative expression units.
*p<0.05; **p<0.01; ***p<0.001 (compared to M-CSF + RANKL condition).

DISCUSSION

ILCs, which comprise a part of the innate immune system, reside mainly in the mucosal area of the human body (26). Recently, ILCs have been identified in the pathogenesis of axSpA, linking these immune cells with dysbiosis (27). Recent studies have shown that gut dysbiosis is common in patients with SpA and could act as an environmental factor in axSpA pathogenesis (28). *Ruminococcus gnavus* was found to be increased in SpA patients with inflammatory bowel disease and even showed a significant correlation with the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) (29). Numerous immune cells, including ILCs, reside in the lamina propria layer of the gastrointestinal tract, and the gut microbiome affects the differentiation of these immune cells (30). Ciccia et al. (8) observed increased IL-22⁺ ILC3 in the peripheral blood, bone marrow, and gut of patients with ankylosing spondylitis, and the gut ILC3 level was significantly correlated with the BASDAI. These ILC3 levels were decreased after tumour necrosis factor-alpha inhibitor treatment (8). Furthermore, IL-22⁺ ILC3 levels in synovial fluid and synovial tissue were significantly elevated in patients with SpA compared to healthy controls (9). IL-22⁺ ILC3 levels were prominently elevated in the synovial tissue of SpA patients relative to the peripheral blood or synovial fluid of identical SpA patients (9). ILC3 can be sub-divided into NKp44-positive and

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-negative cells, and IL-22 can be expressed in both NKp44$^+$ and NKp44$^-$ ILC3 cells, whereas IL-17A is only expressed in the NKp44$^-$ ILC3 population (5). Interestingly, upregulated ILC3 in SpA was dominantly expressed by IL-22 rather than IL-17A (8,9). These results provide new insights into the pathogenesis of SpA and suggest that IL-22$^+$ ILC3 may be the cornerstone pathologic cell type in SpA pathogenesis. In the present study, we demonstrated that circulating IL-22$^+$ ILC3 levels were significantly correlated with disease activity. However, the correlation between circulating IL-22$^+$ ILC3 and ASDAS-CRP was insignificant when dividing axSpA patients into 2 groups according to the presence of dyslipidaemia (**Supplementary Fig. 2**). Furthermore, serum levels of IL-22 did not show significant correlation with ASDAS-CRP (**Supplementary Fig. 3**). These may arise from relative small sample size and low disease activity of enrolled patients (median ASDAS-CRP 1.7). Further study including larger sample size and axSpA patients with high disease activity may clarify the results of present study.

AxSpA patients have an increased risk of cardiovascular disease; therefore, managing dyslipidaemia is important in axSpA patients (31). However, dyslipidaemia not only affects cardiovascular risk but also the inflammatory response and immune cell differentiation. LDL-C elevates the proliferation and mobilisation of hematopoietic stem and progenitor cells of the bone marrow as well as eventually increases circulating and tissue-residing neutrophils and monocytes (17,18). In addition, oxLDL-C activates Ag-presenting cells via toll-like receptors and activates B and T cells in atherosclerosis (32). A recent study showed that oxLDL-C stimulation could alter ILC1 and ILC2 proportions, which implies a potential role of dyslipidaemia in regulating the ILC population (19). Here, we showed that axSpA patients with dyslipidaemia had a higher proportion of circulating IL-22$^+$ ILC3, and oxLDL-C stimulation directly increased IL-22$^+$ ILC3, IL-17A$^+$ NKp44$^-$ ILC3, and IL-17A$^+$ CD4$^+$ T cells. Although the effect of oxLDL-C was not specific for IL-22$^+$ ILC3 differentiation, it still demonstrated a direct effect on pathologic immune cell differentiation. To prove direct effect of oxLDL-C on IL-22$^+$ ILC3 differentiation, we demonstrated oxLDL-C receptor, CD36, expression on ILC3 (**Fig. 3B**). In addition, oxLDL-C induced IL-22$^+$ ILC3 and Th17 differentiation were attenuated by CD36 blocking agent, which implies effect of oxLDL-C was induced by CD36 mediated signal pathway (**Fig. 3A**). These could reinforce the importance of managing dyslipidaemia in axSpA via dual actions: 1) reducing cardiovascular event risk and 2) modulating pathologic immune cells.

Acute inflammatory lesions on spine magnetic resonance images (bone marrow oedema) in axSpA patients usually precede chronic lesions, such as fat metaplasia or syndesmophyte formation (33). The acute inflammatory lesion of the spine showed osteoclast dominance, whereas fat metaplasia showed osteoblast dominance (14). The inflammatory and erosive change of the vertebral corner typically precedes the enchondral ossification and syndesmophyte formation of axSpA; therefore, preventing osteoclast-mediated erosive change may attenuate later syndesmophyte formation. IL-22 is a well-known osteoclast-inducing cytokine in RA (15). In addition, IL-22 produced by ROR-γt$^+$ CD3$^+$ CD4$^-$ CD8$^-$ entheseal resident T cells was associated with osteoblast-mediated new bone formation via STAT3 signalling in an animal model of SpA (34). In several animal model, oxLDL-C increased osteoclast differentiation by increasing expression of RANKL and pro-inflammatory cytokines (IL-1β, IL-6, and TNF) (20,21). Here, we showed that IL-22 stimulation with oxLDL-C promoted the osteoclastogenesis of monocytes derived from axSpA patients. Expression levels of *c-Fos* and *RANK* showed tendency to decrease at low dose IL-22 (10 ng/ml) with oxLDL-C, however these were non-significant. Furthermore, TRAP$^+$ multinucleated cell count was not decreased in oxLDL-C + low dose IL-22, therefore

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these does not support the protective role of oxLDL-C in osteoclastogenesis when low dose of IL-22 was added. In addition, we demonstrated that oxLDL-C and IL-22 increased osteoclastogenesis of monocyte driven from axSpA patients, and this was attenuated by IL-22 blocking agents. These implies potential harmful effects of oxLDL-C and IL-22 on bone homeostasis of axSpA patients, and blocking IL-22 may be useful to regulate oxLDL-C induced osteoclasteogenesis. Also, targeting IL-22 may exert dual therapeutic effects by reducing the osteoclastogenesis of early spinal lesions (e.g., bone marrow oedema and erosive change) and suppressing osteoblast-mediated abnormal bone growth in the vertebral corner (syndesmophyte formation). Further study, investigating *in vivo* effect of oxLDL-C and IL-22 on osteoclastogenesis and IL-22 inhibitor on oxLDL-C induced osteoclastogenesis in real axSpA patients should be performed.

The present study has several limitations. First, the sample size of enrolled axSpA patients was small. Furthermore, the absolute percentage of circulating ILC3 was very low (below 1% of the total PBMCs). However, although the percentage of circulating ILC3 was low, we showed the potential role of circulating IL-22$^+$ ILC3 as a disease activity parameter for the first time. Further studies comparing circulating IL-22$^+$ ILC3 and vertebral corner resident IL-22$^+$ ILC3 as well as larger sample size studies should be performed to reinforce the results of the present work. Second, the present study was performed in a cross-sectional manner, which could not reveal the response of circulating IL-22$^+$ ILC3 after treatment. Comparing circulating IL-22$^+$ ILC3 before and after treatment would provide more evidence regarding the role of IL-22$^+$ ILC3 in axSpA pathogenesis. Third, the underlying mechanism by which oxLDL-C enhances pathologic immune cell differentiation was not identified. Fourth, we showed the potential role of dyslipidaemia in augmenting pathologic immune cell differentiation *in vitro*. The clinical effects of dyslipidaemia treatment on actual axSpA patients were not investigated.

The present study demonstrated the potential role of circulating IL-22$^+$ ILC3 as a disease activity marker in axSpA patients. Furthermore, circulating IL-22$^+$ ILC3 levels were significantly elevated, particularly in axSpA patients with dyslipidaemia, and oxLDL-C enhanced pathologic immune cell differentiation (including IL-22$^+$ ILC3). ILC3-producing cytokines, IL-22, and oxLDL-C enhanced the osteoclastogenesis of axSpA patients.

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SUPPLEMENTARY MATERIALS

Supplementary Figure 1

Gating strategy of CD4$^+$ T cells and ILC3. (A) Th1 (CD4$^+$ IFN-γ$^+$), Th17 (CD4$^+$ IL-17A$^+$), and Th22 (CD4$^+$ IL-22$^+$) cells were analysed using flow cytometry. (B) Flow cytometry gating strategy for IL-17A$^+$ NKp44$^-$ ILC3 (CD3$^-$ CD127$^+$ CD117$^+$ CD294$^-$ IL-17A$^+$ NKp44$^-$). (C) Gating strategy for IL-

22$^+$ ILC3 (CD3$^-$ CD127$^+$ CD117$^+$ CD294$^-$ IL-22$^+$). (D) Flow cytometry image of unstained control of IL-17A$^+$ ILC3. (E) Flow cytometry image of unstained control of IL-22$^+$ ILC3.

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Supplementary Figure 2

Correlation between circulating immune cells and ASDAS-CRP of axSpA without dyslipidaemia (A) and axSpA with dyslipidaemia (B).

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Supplementary Figure 3

Correlation between serum levels of IL-22/IL-17A/TNF-α and ASDAS-CRP.

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Supplementary Figure 4

Effects of oxLDL-C and IL-22 blocking Ab on the osteoclastogenesis of axSpA-derived peripheral blood CD14$^+$ monocytes. Peripheral blood CD14$^+$ monocytes (1×10^6 cells) were obtained from axSpA patients (n=5). (A) These were cultured with oxLDL-C (10 μg/ml) + IL-22 (50 ng/ml) or oxLDL-C (10 μg/ml) + IL-22 (50 ng/ml) + IL-22 blocking Ab (2 μg/ml) containing M-CSF (25 ng/ml) and RANKL (30 ng/ml) and stained for TRAP. Experiment conditions were blinded, and mean TRAP$^+$ multinucleated cells number counted by 3 individuals were used. (B) TRAP$^+$ multinucleated cells were counted. (C) *CTR*, *RANK*, *c-Fos*, *OSCAR*, *NFATc1*, *TRAP*, and *Cathepsin K* mRNA expression levels were measured by real-time quantitative polymerase chain reaction. Data were normalised to beta-actin and reported in relative expression units.

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