Immune cells and bone formation in ankylosing spondylitis

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Abstract

Objectives

The aims of this study were to examine immune cell proportions in peripheral blood of patients with ankylosing spondylitis (AS) and to investigate relationships between immune cells, level of bone formation related molecules, and radiographic changes.

Methods

Forty-nine AS patients and 53 age- and sex-matched healthy controls (HCs) were enrolled in this study. Clinical parameters were extensively evaluated in the study subjects. CD4+ T-cells, CD8+ T-cells, CD56+ T-cells, natural killer cells, and natural killer T (NKT) cells in peripheral blood were measured by flow cytometry. Serum levels of Dickkopf-1 and bone morphogenic proteins were determined using enzyme linked immunosorbent assays. Modified Stokes AS spinal scores were used to assess radiographic changes.

Results

Patients were found to have a significantly higher percentages of CD56+T-cells than healthy controls (median 1.31% vs. 0.53%, $p<0.001$), whereas percentages of peripheral blood natural killer T (NKT) cell were lower in patients than in controls (median 0.07 % vs. 0.10%, $p=0.010$). Moreover, mean CD 56+T to NKT cell ratio was markedly higher in patients. Although no significant correlations were observed between the immune cell percentages and bone formation-related molecule levels, interestingly, patients with a higher CD56+T to NKT cell ratio at baseline were found to develop greater radiographic changes ($r=0.79$, $p=0.007$, age and disease duration adjusted) during 3 years of radiographic follow-up.

Conclusion

An altered T-cell compartment, particularly with respect to CD56+ T and NKT cells, was observed in AS patients and could contribute to radiographic changes in AS.

Key words

ankylosing spondylitis, CD56+ T-cell, natural killer T-cell, mSASSS

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Introduction

Ankylosing spondylitis (AS) is the major subtype of a group of chronic inflammatory diseases, known as seronegative spondyloarthritis (1). Clinically, the main features of AS are inflammatory back pain and inflammation at other locations in the axial skeleton, peripheral arthritis, enthesitis, and anterior uveitis (2, 3). The characteristic feature of AS is extensive new bone formation throughout the spine (1).

Regarding bone formation, bone morphogenic proteins (BMPs) have been implicated in the synthesis of bone matrix (4). In biopsies of early-stage Achilles tendon enthesopathy, several BMPs have been reported to be expressed at sites of ossifying enthesitis (5), and it has been demonstrated that BMP activity increases during enthesophyte formation in AS (6, 7). Dickkopf-1 (DKK-1) is a natural inhibitor of Wnt and actively prevents new bone formation (8, 9), and it has been reported that DKK-1 levels in AS are substantially lower than in healthy population (10). Furthermore, it has been reported that activated T-cell cytokines induce BMP-2 (11).

In terms of the presence of chronic inflammation in AS, direct and indirect evidence suggests that T-cells play a role in its pathogenesis. $CD8^+$ T-cells are present at the leading edge of the cellular infiltrate during enthesitis (12). In addition, it has been demonstrated that inflammatory disease in B27 transgenic rats (a disease specific animal model) is T-cell-dependent (13). Furthermore, an impaired Th1 cytokine response might be of pathophysiological significance, and could be associated with immune effectors, such as, $CD4^+$T, $CD8^+$T, and natural killer (NK) cells (14, 15).

Although the proportions of peripheral blood immune cells have been evaluated in AS (16-18), much less has been known about the relations between immune cells and bony changes in AS. In joint diseases, interactions between Wnts and BMPs are complex, because the specific cascades involved are influenced by the presence of proinflammatory cytokines, and inflammatory and regulatory cells (19). Therefore, the aim of this study was to determine immune cell proportions in the peripheral blood of AS patients and whether immune cells are associated with bone formation related molecules or radiographic changes.

Materials and methods

Study Subjects

Forty-nine AS patients and 53 age- and sex-matched healthy controls (HCs) were enrolled in the present study. All the patients satisfied the modified New York criteria for AS (20). Patients with a history of neoplasm, a recent acute infection, or a history of any other chronic inflammatory disease were excluded from the study. HCs were screened by questionnaire to exclude those with a personal or family history of arthritis. The study was approved by the Institutional Review Board of Chonnam National University Hospital.

Clinical assessments

Clinical parameters were extensively evaluated in all the study subjects. Clinical data included age, sex, duration of disease, disease specific clinical symptoms, family history of AS, medication, and HLA-B27 carrier status. In addition, Bath ankylosing spondylitis disease activity indices (BASDAI) (21), Bath ankylosing spondylitis functional indices (BASFI) (22), and Bath ankylosing spondylitis metrology indices (BASMI) (23) were determined. Modified Stokes ankylosing spondylitis spinal scores (mSASSS) (24), which have been reported to be the most sensitive for evaluation of spinal changes in AS (25), were used to assess radiographic damage. The vertebral images of poor quality and those not properly captured were excluded.

Measurements of serum DKK-1, BMP-2, BMP-6 and BMP-7 levels

Venous blood samples were collected from all the AS patients at the same time as clinical parameters were measured. Serum specimens were separated by centrifugation at 1500 revolutions per minute (rpm) for 10 min, and stored at -80°C until assayed. Serum levels of DKK-1, BMP-2, BMP-6 and BMP-7 were measured using commercial en-

zyme linked immunosorbent assay kit (R&D systems Inc., Minneapolis, MN, USA). All the assays were performed in duplicate.

Isolation and flow cytometry analysis of peripheral blood mononuclear cells (PBMCs) and monoclonal antibodies

Peripheral venous blood samples were collected into tubes containing heparin, and PBMCs were isolated by density-gradient centrifugation using Ficoll-Paque Plus solution (d=1.077 gm/ml; Amersham Bioscience, Uppsala, Sweden). The following monoclonal antibodies (mAbs) and reagents were used in this study: Peridinin chlorophyll-*a* protein (PerCP)-conjugated anti-CD3 mAb, FITC-conjugated anti-CD4 mAb, allophycocyanin (APC)-conjugated anti-CD8 mAb, allophycocyanin (APC)-conjugated anti-CD56 mAb, and PE-conjugated 6B11 mAb (Beckman Coulter, Marseille, France). Cells were stained with combinations of appropriate mAbs at 4°C for 20 minutes. Stained cells were then analysed on a FACSCalibur flow cytometer using Cell Quest software (BD Biosciences, Mountain View, CA, USA). NK cells were phenotypically identified as CD3-CD56$^+$ cells by flow cytometry (16). The 6B11 mAb recognises all T-cells expressing the invariant TCR α chain, and can be used in combination with anti-Vα24, anti-Vβ11, or anti-CD3 for the detection of natural killer T (NKT) cell with high specificity and sensitivity (26-28). Thus, based on flow cytometry, NKT cells were phenotypically identified as CD3$^+$6B11$^+$ cells (28).

Statistical analysis

Statistical analysis was performed using the Student's *t*-test, and the Kruskal-Wallis nonparametric test. Pearson's correlation test was used to establish the presence of correlations. The statistical analyses were performed using SPSS version 17.0 software (SPSS, Chicago, IL), and *p*-values of less than 0.05 were considered statistically significant.

Results

The baseline characteristics and bone biomarkers of the study subjects are summarised in Table I. Mean age (SD) of the AS patients was 36.4 (10.8) years; 89.7% were men and mean disease duration was 4.4 (4.2) years. Low back pain was the most common clinical symptom (71%) in AS patients, followed by hip pain (63%) and buttock pain (57%). HLA B27 was present in 40 patients (95%). TNF blockers and sulfasalazine was treated in thirty (61.2%) and twenty six patients (53.1%), respectively. It was previously reported that DKK-1 levels in AS patients were substantially lower than in HCs (10). However, in the present study, mean serum DKK-1 level (S.E.M) in AS patients was 341.8±55.1 pg/ml, which was similar to the serum levels of controls (mean±S.E.M. 239.6±32.4 pg/ml). No significant difference was observed between patients and controls in terms of bone biomarker levels. We further sought to investigate whether anti-TNF agent and sulfasalazine had any effect on serum bone formation related mole-

Table I. Clinical and laboratory characteristics of patients and HCs.

	HCs	AS
Total number	53	49
Age, mean±SD (years)*	34.9±9.0	36.4±10.8
Male, n (%)*	47 (88.7)	44 (89.7)
Disease duration (years), mean±SD	NA	4.4±4.2
Disease specific clinical symptom		
LBP, n (%)	NA	35 (71)
hip pain, n (%)	NA	31 (63)
buttock pain, n (%)	NA	28 (57)
neck stiffness, n (%)	NA	27 (55)
peripheral arthritis, n (%)	NA	26 (53)
enthesitis, n (%)	NA	12 (24)
eye involvement, n (%)	NA	13 (16)
Family history, n (%)	NA	3 (6)
Current medications		
NSAID, n (%)	NA	40 (82)
sulfasalazine, n (%)	NA	26 (53.1)
anti-TNF therapy, n (%)	NA	30 (61.2)
HLA B27, n (%)	NA	40 (95)
BASDAI, mean±SD	NA	3.0±2.0
BASFI, mean±SD	NA	2.3±2.2
BASMI, mean±SD	NA	2.9±2.0
ESR, mean±SD (mm/hr)	NA	28.4±22.0
CRP, mean±SD (mg/dL)	NA	0.8±0.8
DKK-1, mean±SEM (pg/ml)*	239.6±32.4	341.8±55.1
BMP-2, mean±SEM (pg/ml)*	31.7±3.8	35.1±3.1
BMP-6, mean±SEM (pg/ml)*	101.2±27.9	81.6±36.9
BMP-7, mean±SEM (pg/ml)*	19.3±2.1	8.6±1.3

*Statistically not significant; HCs: healthy controls; NA: not applicable; LBP: low back pain; NSAID: non-steroidal anti-inflammatory drug; TNF: tumour necrosis factor; BASDAI: Bath ankylosing spondylitis disease activity index; BASFI: Bath ankylosing spondylitis functional index; BASMI: Bath ankylosing spondylitis metrology index; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; DKK-1: Dickkopf-1; BMP: bone morphogenic protein; SEM: standard error of mean.

Table II. Comparative analysis of immune cells percentages in peripheral blood of patients and controls.

Subtype of immune cell (%)	HCs (n=53)	AS (n=49)	*p*-value
T-cell, median (25th–75th)	66.1 (60.4–73.5)	63.9 (54.8–71.7)	NS
CD4$^+$ T-cell, median (25th–75th)	37.3 (30.3–44.9)	35.5 (30.6–42.9)	NS
CD8$^+$ T-cell, median (25th–75th)	23.5 (19.7–30.4)	22.3 (17.5–26.9)	0.037
CD56$^+$ T-cell, median (25th–75th)	0.53 (0.31–1.00)	1.31 (0.59–2.43)	<0.001
NKT cell, median (25th–75th)	0.10 (0.07–0.11)	0.07 (0.05–0.08)	0.010
NK cell, median (25th–75th)	15.7 (10.2–23.4)	14.0 (9.52–18.76)	NS

Natural killer (NK) and NKT cells were identified as CD3-CD56$^+$ and CD3$^+$6B11$^+$ cells by flow cytometry, respectively. NS: not significant; HC: healthy controls.

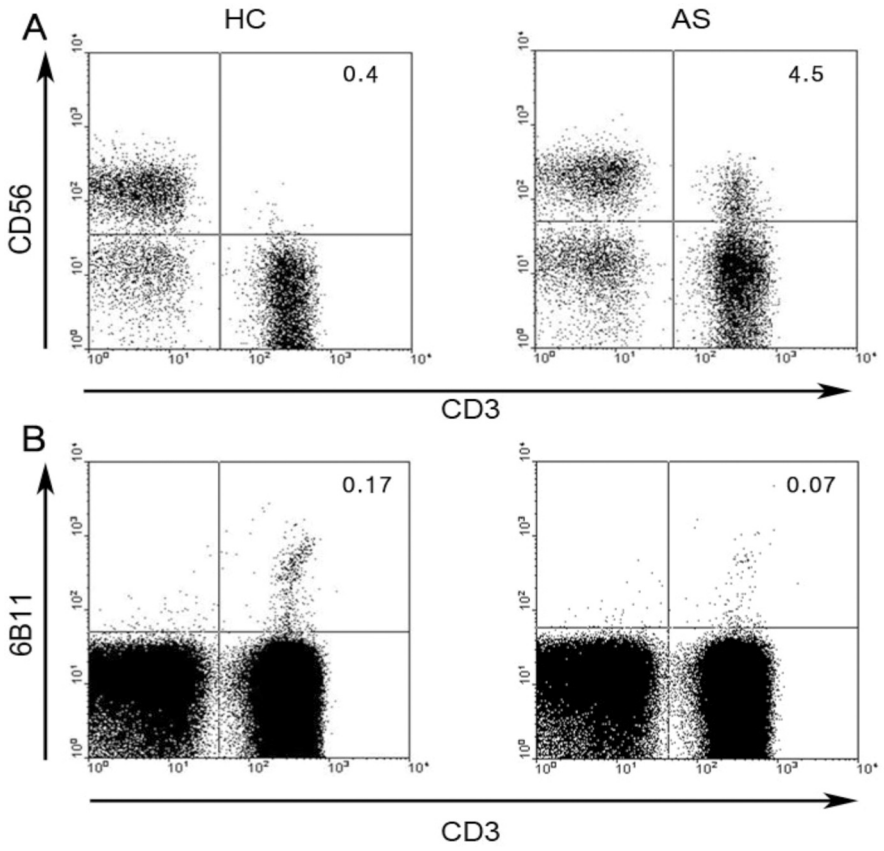

Fig. 1. Representative dot-plot diagrams of peripheral blood cells stained with anti-CD3, anti-CD56, and anti-6B11 monoclonal antibodies.
A $CD56^+$T-cell proportions were increased in patient peripheral blood, **B** whereas NKT cell proportions were reduced. NKT cells were identified as $CD3^+6B11^+$ cells by flow cytometry.

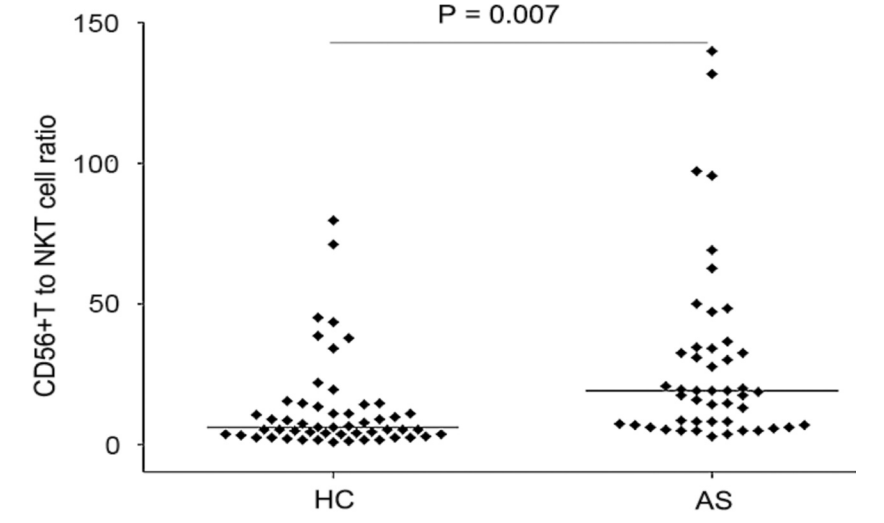

Fig. 2. $CD56^+$ T to NKT cell ratios were elevated in AS patients. Horizontal bars indicate medians.

cules. Patients with medications had similar serum bone biomarker levels when compared with the ones without medications (data not shown).

The percentages of immune cells in peripheral blood samples were determined in all the 102 study subjects (Table II). Patients were found to have a significantly greater percentage of $CD56^+$T-cells than controls (median 1.31% *vs.* 0.53%, $p<0.001$). On the other hand, peripheral $CD8^+$T-cell and NKT percentages were lower in patients (median 22.3 % *vs.* 23.5, $p=0.037$; median 0.07% *vs.* 0.10%, $p=0.010$). Representative flow cytometric analyses of $CD56^+$T and NKT cells are shown in Figure 1. In addition, the mean $CD56^+$T to NKT cell ratio was significantly higher in patients (Fig. 2, median 19.5 *vs.* 5.3, $p=0.007$). Although we investigated for the associations between immune cell percentages and clinical variables such as, age, specific symptoms, HLA B27 positivity, BASMI, BASDAI, BASFI, and current medications, no relations were observed (data not shown).

The results of correlation analysis conducted to examine the relationship between immune cells percentages and levels of bone-formation related molecules in peripheral blood are presented in Table III. However, no significant correlation was found between immune cell percentages and the levels of any bone formation-related molecules.

Since the $CD56^+$T to NKT cell ratio was significantly higher in patients, we further examined the clinical relevance of the cell ratios. During a 3-year follow-up, we found that patients (n=21) with a higher $CD56^+$T to NKT cell ratio at baseline developed significantly greater radiographic changes (Fig. 3, $r=0.79$, $p=0.007$, age and disease duration was adjusted).

Discussion

Regarding T-cell subsets, the distributions of $CD4^+$T and $CD8^+$T cell subsets in peripheral blood differ in accordance with their expressing cytokines among AS studies (18, 29, 30). Szanto *et al.* reported that $CD4^+$T-cell percentages was significantly higher in AS patients (18), which was not the case in the present study. Other authors reported that the percentage of $IL4^+CD8^+$ cells was elevated in patients (31, 32), but the percentage of $CD8^+$T-cell was similar in AS patients and in HCs (18). In the present study, the percentage of $CD8^+$T-cell was found to be lower in all the 49 patients than in any of the 53 age-matched HCs. It has been suggested that the recruitment of $CD8^+$T-cells at sites of inflammation might play an important role in damage of joints in association with AS (33), which might be related to the concomitant migration of $CD8^+$ lymphocytes to the affected joints. This

Table III. Correlation analyses for relations between percentages of immune cells and bone formation related molecules levels in the peripheral blood of AS patients.

		Dkk-1	BMP-2	BMP-6	BMP-7
Total T-cell	Pearson correlation	-0.097	0.070	-0.105	0.215
	Sig. (2-tailed)	0.508	0.633	0.473	0.139
	n	49	49	49	49
CD4+T-cell	Pearson correlation	-0.056	0.071	-0.043	0.218
	Sig. (2-tailed)	0.701	0.626	0.770	0.132
	n	49	49	49	49
CD8+T-cell	Pearson correlation	-0.022	-0.084	-0.114	0.137
	Sig. (2-tailed)	0.880	0.564	0.434	0.348
	n	49	49	49	49
CD56+T-cell	Pearson correlation	-0.031	0.121	-0.108	0.027
	Sig. (2-tailed)	0.831	0.409	0.459	0.853
	n	49	49	49	49
NKT cell	Pearson correlation	-0.092	0.191	-0.162	-0.074
	Sig. (2-tailed)	0.532	0.190	0.267	0.612
	n	49	49	49	49
NK cell	Pearson correlation	0.188	0.011	0.126	-0.095
	Sig. (2-tailed)	0.197	0.941	0.388	0.516
	n	49	49	49	49
CD56+T/NKT ratio	Pearson correlation	-0.008	0.114	-0.075	0.018
	Sig. (2-tailed)	0.958	0.437	0.610	0.903
	n	49	49	49	49

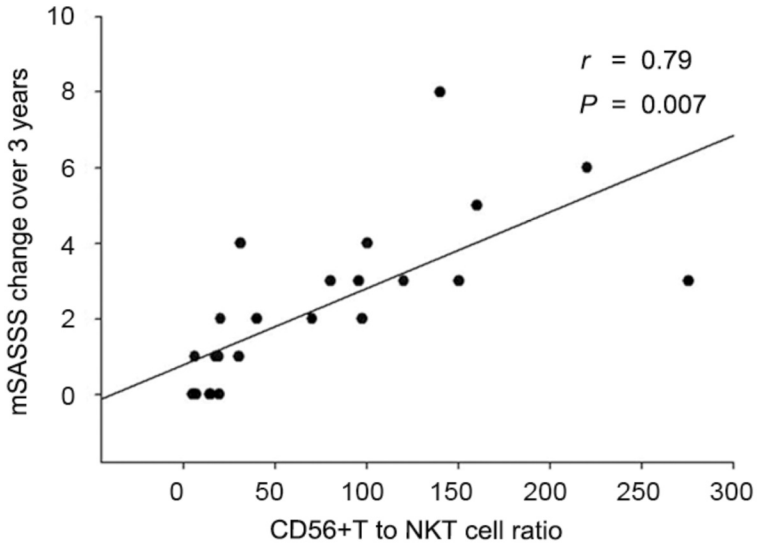

Fig. 3. Radiographic changes as determined by mSASSS over a 3-year follow-up in patients with AS according to CD56+T to NKT cell ratio at baseline (age and disease duration adjusted).

recruitment may be responsible for the presence of lower levels of CD8+T-cells in peripheral blood, as observed in the present study. It has been previously reported that AS patients have significantly higher percentages of NK cells in peripheral blood than in HCs (34). However, in this respect, we observed no differences between patients and HCs (16).

Human NKT cells are a distinct subset of T lymphocytes and are characterised by the restricted expression of an invariant Vα24-Jα18 T-cell receptor (TCR) chain paired with the Vβ11 TCR chain, which recognises glycolipid antigens, such as α-galactosylceramide (α-GalCer), presented by the MHC class I-like molecule CD1d (35). NKT cells have been implicated in protection against a wide range of autoimmune diseases, for example, in an *in vivo* study; repeated administrations of α-Galcer analogue led to increased synthesis of Th2 cytokine mediated by NKT cells (36, 37). CD56+T-cells are a unique subset of human T-cells and co-express T-cell receptor complex (CD3) and NK receptors (CD56). CD56+T-cells are important components of immune responses to infectious agents (38), tumour rejection (39, 40) and autoimmunity (41). Furthermore, their levels have been reported to be elevated in peripheral blood in the presence of malignancies and to increase with age (42, 43), and to be significantly diminished in the peripheral blood of patients with rheumatoid arthritis and psoriatic arthritis (44, 45). In Behçet's uveitis, CD56+T-cell proportions were found to be significantly elevated in aqueous humour and peripheral blood, which suggests that human CD56+T-cells play an important role in Th1 responses and that they may harm the target organs (46). In the present study, percentages of CD56+T-cells were found to be significantly elevated in AS along with a significant decrease in the percentages of NKT cells. Moreover, the CD56+T to NKT cell ratio was found to be significantly higher in AS patients than in HCs. These observations suggest that both CD56+T and NKT cells play important roles in the pathogenesis of AS.

To examine the clinical relevance of CD56+T and NKT cells, we investigated the associations between the proportions of these immune cells in peripheral blood and clinical variables. Unfortunately, no evidence with respect to connection between immune cell proportions and clinical parameters was found. The clear evidence regarding the lower and higher proportions of NKT and CD56+T-cells in this study raises the question as to whether the higher CD56+T/NKT cell ratio in patients might be attributable to the new bony formation. Although only a small number of patients were followed for 3 years, it was found that CD56+T/NKT cell ratio at baseline predicted radiographic changes. However, no relationship was found between the bone biomarkers and immune cells. We suggest the following explanations for the discrepancies between bone biomarkers and radiographic changes, related to CD56+T/NKT cell ratio. Despite the fact that balance in the regulation of bone formation and resorption is es-

sential in bone remodelling, we did not evaluate the level of bone resorption markers. Among bone formation related markers, only DKK-1 and BMPs were selected for this project.

Before confirming these results, we should keep in mind when interpreting the outcome. First, the patients with 3-year follow-up radiographs were few in number (n=21) to represent the result in all the patients with AS. For accurate analysis, large scaled subsequent studies are necessary to validate the results. Second, bone remodelling depends on the spatial and temporal coupling of bone formation by osteoblasts and bone resorption by osteoclasts. Therefore, it is inevitable that the molecular based mechanical study of such interactions among the cells should be followed. A recent study has demonstrated that activated NKT cells were involved in the function of osteoclasts (47). Thus, further functional studies on such T-cell subsets are required to establish their roles in bony changes of AS.

In summary, the present study shows that the levels of $CD56^+$T cell and NKT cell were altered in AS patients. Furthermore, $CD56^+$T/NKT cell ratio was found to predict radiographic changes in AS.

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