Endothelial dysfunction is independent of inflammation and altered CCR7 T cell expression in patients with ankylosing spondylitis

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Abstract

Objective

The accumulation of CCR7 (chemokine receptor 7) positive cells in the vessel wall may be involved in endothelial dysfunction and subsequent accelerated atherogenesis. CCR7 plays a crucial role in T cell and monocyte migration / homing and in priming of naive T lymphocytes in non-lymphoid tissues in chronic inflammatory diseases.
Our objective was to investigate the endothelial function and inflammation-driven expression of CCR7 on T lymphocytes in patients with ankylosing spondylitis (AS).

Methods

We performed flow cytometry to assess the distribution of peripheral blood T cell subpopulations in the context of serum inflammatory markers (TNF-α, IL-6, sICAM-1) and asymmetric dimethylarginine (ADMA) in 38 patients with AS with active disease, and in 20 healthy controls.

Results

Patients with AS demonstrated higher ADMA (0.74±0.2 μmol/l vs. 0.64±0.15 μmol/l; p=0.03), as well as elevated inflammatory markers (TNFα, IL-6, sICAM-1) and increased proportions of circulating CCR7-positive lymphocytes largely attributable to elevated $CD8^+$ naive T cells (47.1±17 vs. 34.3±13.1%; p=0.005).
However, ADMA did not correlate with either CCR7-positive lymphocytes or inflammatory markers.

Conclusion

We found an increased percentage of peripheral CCR7 T cells accompanied by endothelial dysfunction in patients with AS. The lack of direct associations between ADMA and inflammation may suggest the presence of other pathogenic mechanisms contributing to accelerated atherogenesis and increased cardiovascular risk in AS.

Key words

ankylosing spondylitis, CCR7 receptors, endothelium, lymphocytes, asymmetric dimethylarginine, inflammation

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Introduction

Ankylosing spondylitis (AS) is a chronic inflammatory disease associated with increased cardiovascular morbidity and mortality (1), which is related not only to traditional cardiovascular risk factors but also to systemic inflammation. According to EULAR (European League Against Rheumatism) guidelines on cardiovascular disease (CVD) risk assessment in patients with inflammatory arthritis, AS should be regarded as a condition associated with higher risk for CVD, although the evidence for the relationship between inflammation and enhanced CVD risk is less evident compared with RA (2). Additionally, disease-modifying anti-rheumatic drugs may decrease CVD risk.

Inflammation contributes to the development of endothelial dysfunction, an antecedent of atherosclerotic plaques and it is associated with increased levels of proinflammatory cytokines, prothrombotic factors and enhanced expression of adhesion molecules, as well as accelerated migration of T cells into the vessel wall. Chemokine receptor 7 (CCR7) is expressed on naive and central memory T cells and it is known to be involved in controlling T cell activation, migration and homeostasis. Moreover, CCR7 regulates T cell homing to lymph nodes. During chronic inflammation the ectopic lymphoid tissue localised at sites of inflammation also enhances the accumulation of T cells through CCR7 signalling (3). Furthermore, CCR7 is expressed in human atherosclerotic plaques (4) and CCR7-dependent circulation of T cells may contribute to the maintenance of the immune response in atherosclerosis (5).

However, results of studies on the role of CCR7 in atherosclerosis remain inconsistent and the expression of CCR7 on peripheral lymphocytes in AS have not been reported so far. Our aim was to evaluate the proportion of circulating lymphocyte T subsets according to their CCR7 expression and to assess the relationship between T cell phenotype and biochemical markers of inflammation and endothelial dysfunction in AS patients with active disease.

Materials and methods

Patients

A total of 38 adult patients with AS, meeting the modified New York criteria (6) with active disease were recruited through the Rheumatology Clinic at the University Hospital in Krakow and 20 healthy patients as a control group. Disease activity was assessed using the ASDAS (Ankylosing Spondylitis Disease Activity Score), which in addition to clinical parameters also includes acute phase reactants (7). Patients with a history of coronary heart disease, heart failure, stroke, transient ischaemic attack, diabetes, chronic kidney disease, malignancy, acute infection within the 4 preceding weeks and treated with disease-modifying anti-rheumatic drugs were excluded. Treatment for AS included non-steroidal anti-inflammatory drugs. All procedures performed in the study were in accordance with the ethical standards of the institutional research committee (Jagiellonian University Ethical Committee). Informed consent was obtained from all the participants included in the study.

Study protocol

Blood was processed within 2 hours of collection. Serum was stored in -80ºC until further analysis and EDTA-anticoagulated blood was immediately transported to laboratory for flow cytometric determinations. Demographic, clinical characteristics and anthropometric data were assessed. Blood pressure measurements were taken using oscillometric device (Omron M2, Omron Healthcare, Hoofddorp, The Netherlands) on the left arm after 5 minutes rest in a sitting position, second measurement was recorded.

Biochemical assays

Serum high sensitivity C-reactive protein (hs-CRP) was analysed by immunonephelometry (Dade Behring II, Ramsey, MN, USA). Glucose, lipids and creatinine were analysed using standardised laboratory techniques (Hitachi 917 chemistry analyser, Roche Diagnostics, Holliston, MA, USA).

ELISA

Human TNF-α, IL-6, sICAM1/CD54

(R&D Systems, Inc, Minneapolis, MN, USA), ADMA, SDMA (DLD Diagnostika, GmBH, Hamburg, Germany) levels were determined using commercial kits.

Flow cytometry

Peripheral blood mononuclear cells were isolated from EDTA-anticoagulated blood and collected on the same day as remaining samples, using density gradient centrifugation and LSM 1077 (PAA Laboratories GmbH, Pasching, Austria). One hundred thousand cells were stained for 20 minutes with fluorochrome-conjugated mouse anti-human monoclonal antibodies: CD45RO-PE, CD45RA-FITC, CD3-PerCP, CD4-APC, CD8-APC-H7, CCR7-PE-Cy7 (purchased from BD Biosciences, Diego, CA, USA) and then washed with phosphate buffered saline containing 1% fetal bovine serum (GIBCO, Invitrogen LTD, Paisley, UK). The cells were processed using the FACSCanto II flow cytometer (BD Biosciences), and then analysed with FACSDiva (BD Biosciences) and FlowJo (Tree Star Inc., Ashland, OR, USA) software. The lymphocytes were gated in a forward/side (FSC/SSC) scatter and analysed for CD3 expression. Lymphocyte subsets were defined according to CD4 and CD8 expression as T helper ($CD4^+CD8^-$) and T cytotoxic ($CD4^-CD8^+$) lymphocytes. T helper and T cytotoxic lymphocytes were analysed according to CD45RA and CCR7 expression and defined as $CCR7^+$T cells: naïve ($CD45RA^+CCR7^+$) and central memory ($CD45RA^-CCR7^+$), $CCR7^-$T cells: effector memory ($CD45RA^-CCR7^-$) and terminally differentiated effector memory TEMRA ($CD45RA^+CCR7^-$) lymphocytes. The results have been presented as the percentage of cells expressing particular markers. All experiments were performed under standardised experimental conditions, including the procedure of cell isolation and reagents for sample preparation. The same number of cells and the same amounts of antibodies were used for samples. BD Cytometer Setup for BD FACSDiva using Tracking Beads was performed regularly to ensure operational stability, so that the individual measurements did not differ significantly. Flow cytometry compensation was verified using cells studied in the experiment and BD Multicolor Comp Beads (BD Biosciences). One operator, blinded for clinical characteristics, performed the sample collection and analysis.

Table I. Demographics and clinical characteristics of patients in the study.

	AS (38)	C (20)	*p*-value
Age, yrs	33.6 ± 6,2	34.2 ± 6,5	0.73
Female, %	10.5	30	0.07
Symptom duration, yrs	6.5 (4-10)	NA	NA
SBP, mmHg	120.5 ± 8.5	114.5 ± 13.4	**0.04**
DBP, mmHg	77.8 ± 8.5	77.9 ± 6.7	0.98
BMI, kg/m^2	24.5 ± 3	23.6 ± 2.4	0.23
TC, mmol/l	5.1 ± 1.2	5.2 ± 0.7	0.67
LDL, mmol/l	3.2 ± 1	3.4 ± 0.7	0.48
HDL, mmol/l	1.4 ± 0.3	1.7 ± 0.5	**0.02**
Smoking, %	34.2	20	0.36
NSAID, %	84.2	NA	NA
ASDAS CRP	2.9 ± 0.9	NA	NA
Lymphocyte, %	27 ± 7.3	35.8 ± 0.5	**<0.001**
Creatinine, µmol/l	66 ± 14.2	72 ± 10.2	0.11
Glucose, mmol/l	4.7 ± 0.6	4.8 ± 0.5	0.45

Data are presented as means (±SD), medians (interquartile range) or percentages. NA: not applicable; C: controls; SBP: systolic blood pressure; DBP: diastolic blood pressure; BMI: body mass index; TC: total cholesterol; LDL: low density cholesterol; HDL: high density cholesterol; NSAID: non-steroidal anti-inflammatory drugs; ASDAS: ankylosing spondylitis disease activity score; CRP: C-reactive protein.

Table II. Inflammatory and endothelial markers in patients in the study.

	AS (38)	C (20)	*p*-value
ESR, mm/h	21 (10-40)	4 (2-7)	**<0.001**
CRP, mg/l	5.2 (3.4-15.3)	0.57 (0.3-1.1)	**<0.001**
ADMA, umol/l	0.74 ± 0.2	0.64 ± 0.15	**0.03**
SDMA, umol/l	0.6 ± 0.22	0.62 ± 0.21	0.6

Data are presented as means (±SD) or medians (interquartile range). C: controls; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; ADMA: asymmetric dimethylarginine; SDMA: symmetric dimethylarginine.

Statistical analysis

Statistical analyses were performed using Statistica data analysis software system (v. 10.0.1011.0; StatSoft Inc. Tulsa, USA). Data are presented as means (standard deviation, SD) or medians (interquartile range, IQR) for continuous variables and numbers (percentages) for categorical variables. The accordance with a normal distribution was tested by the Kolmogorov-Smirnov test and uniformity of variances (homoscedasticity) by Leven's test. Intergroup differences were assessed with the unpaired 2-sided Student's *t*-test or Mann-Whitney *U*-test for continuous variables and Fisher's exact test for proportions. Analysis of covariance (ANCOVA) was applied to confirm intergroup differences after controlling for the effect of age. A p-value <0.05 was considered significant. Bivariate correlations between continuous variables were estimated by Pearson's correlation coefficients or Spearman's correlation coefficients (r).

Results

The demographic and clinical characteristics of the patients and controls are presented in Table I. AS patients had increased systolic blood pressure and decreased HDL cholesterol in comparison to the control patients. The percentage of AS patients with hypertension, hypercholesterolaemia, smoking and overweight patients was comparable with the control group (data not shown).

The mean AS activity according to ASDAS calculator was high 2.9 (±0.9). The degree of systemic inflammatory activity assessed with ESR and CRP

was increased in AS (Table II) and inflammatory and endothelial activation markers: TNF-α (1.8 [1.6-2.1] *vs*. 1.4 [1.3-1.5] pg/ml; $p<0.001$), IL-6 (3.6 [2-5.7] *vs*. 0.7 [0.6-1] pg/ml; $p<0.001$) and sICAM-1 (270±62 *vs*. 215±58 ng/ml; $p=0.002$) were elevated in AS patients in comparison to healthy controls (Fig. 1). IL-6 concentration correlated with systemic parameters of disease activity in AS: ASDAS ESR (r=0.42; $p=0.01$), ASDAS CRP (r=0.61; $p<0.001$). In addition, patients with spondyloarthropathy had elevated serum ADMA: 0.74±0.2 μmol/l *vs*. 0.64±0.15 μmol/l; ($p=0.03$) as compared to the control group.

There was a significant decrease in total lymphocyte percentage in AS patients when compared to controls. However, the proportion of circulating T lymphocytes (CCR7$^+$ CD3$^+$) was increased in AS. That finding was attributable to elevated CCR7$^+$ CD8$^+$ T cells in comparison with healthy subjects: 52±16.9% *versus* 40.2±12.6% ($p=0.008$), due to an elevated percentage of naive CD8$^+$ lymphocytes: 47.1±17.1% *vs*. 34.3±13.1% ($p=0.005$) and decreased median (IQR) percentage of terminally differentiated effector memory (TEMRA) CD8$^+$ lymphocytes 13.2 (7.7-21.9) % *vs*. 22.4 (13.4-31.5) % ($p=0.02$). The significance of these differences was maintained upon adjustment for subjects' age by ANCOVA ($p=0.01$ for either CCR7$^+$CD8$^+$ or naïve CD8$^+$ T cells). We did not find any significant correlations between ADMA and percentage of CCR7-positive T cells (r=-0.15; $p=0.37$), as well as ADMA and disease activity (r=0.13; $p=0.45$), ESR (r=-0.11; $p=0.51$), hsCRP (r=0.03; $P=0.86$), TNF-α (r=0.12; $p=0.48$), IL-6 (r=-0.01; $p=0.94$) and ICAM-1 (r=0.1; $p=0.57$). Similarly, T lymphocytes expressing CCR7 did not correlate with any of assessed inflammatory markers (r=-0.05, $p=0.77$ for TNF-α; r=0.19, $p=0.26$ for IL-6 and r=-0.14, $p=0.40$ for sICAM-1).

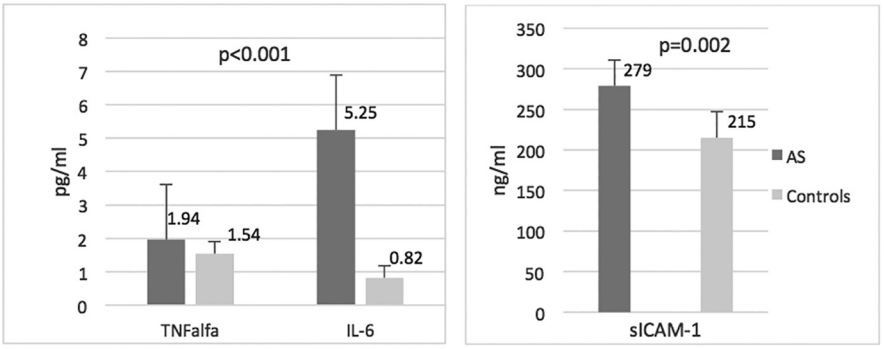

Fig. 1. Assessment of serum inflammatory cytokines in AS patients *versus* controls. Data are presented as means (±SEM). Intergroup differences were assessed by the unpaired 2-sided Student's *t*-test (sICAM-1) and Mann Whitney *U*-test (TNF-α and IL-6).

Table III. CCR7 expression on T cells in patients in the study.

	AS (38)	C (20)	*p*-value
CCR7$^+$CD3$^+$, %	63 ± 12.1	54.4 ± 10.2	**0.01**
CCR7$^+$CD4$^+$, %	74.4 ± 11.7	72.8 ± 9.4	0.6
naive,%	43.2 ± 16.3	40.8 ± 12.3	0.56
central memory, %	31.2 ± 9.8	32 ± 9.2	0.75
CCR7$^+$CD8$^+$, %	52 ± 16.9	40.2 ± 12.6	**0.008**
naive, %	47.1 ± 17	34.3 ± 13.1	**0.005**
central memory,%	4.9 ± 2.5	5.9 ± 3.2	0.16

Data are presented as means (± SD).

Discussion

In this study we assessed the distribution of peripheral T lymphocytes expressing CCR7 and evaluated the relationship between markers of endothelial activation and dysfunction and T cell phenotype in AS patients with active disease. We found increased plasma ADMA and an elevated percentage of CCR7-expressing T cells mostly due to a higher proportion of naive CD8$^+$ cells in these patients.

ADMA is an endogenous inhibitor of NO formation and is associated with cardiovascular risk factors, endothelial dysfunction, subclinical atherosclerosis and adverse cardiovascular events. Nitric oxide (NO) is a co-player in a corollary of multiple pathways governing cell adhesion to vascular endothelia. In animal models of hypercholesterolemia NO decreases leukocyte adhesiveness (8). Inhibition of endothelial NO synthase (eNOS) might contribute to enhanced adhesion of T cells to endothelium. Chan *et al*. observed augmented endothelial adhesiveness of monocytes and T lymphocytes after previous exposure of endothelial monolayers to ADMA (asymmetric dimethylarginine) (9). Increased ADMA in AS patients have been previously demonstrated (10, 11). In the present study we also observed elevated TNF-α concentration in patients with active AS. Thus, an increase in ADMA may be attributable to the TNF-α induced inhibition of the activity of dimethylarginine dimethylaminohydrolase (DDAH), an enzyme which is involved in the degradation of over 80% of ADMA (12). There are experimental data indicating the lack of direct link between ADMA and endothelial vasodilatatory capacity related to endothelial dysfunction, implicating specific functions of DDAH isoforms (13), but increased ADMA levels might facilitate atherogenesis on a long-term basis. The potential reduction in NO synthesis resulting from elevated plasma ADMA together with increased soluble ICAM-1 in our group may favour T cell adhesiveness. After CCR7 stimulation, the affinity of the ICAM-1 ligand LFA-1 (lymphocyte function associated-antigen) on T cells increases, which enhances interactions of T cells with ICAM-1 expressed on the endothelium (14). Thus, it can be hypothesized that elevated concentrations of ADMA and sICAM-1 might reflect enhanced trafficking of monocytes and T cells into the inflamed synovium and possibly also the vascular wall. Additionally, higher ADMA levels could

preferentially favour an accumulation of naive T cells in sites of inflammation, while the CCR7-dependent chemotaxis stimulates not only migration from blood but also the exit from atherosclerotic plaques into lymph nodes along with a gradient of CCL19 and CCL21.

CCR7 is expressed on naive and central memory T cells and regulates T cell homing to lymph nodes. Naive T cells have a high proliferative potential and recirculate through secondary lymphoid organs and tertiary lymphoid tissues, such as sites of chronic inflammation. Memory T cells are a heterogeneous population consisting of two subsets: central memory and effector memory lymphocytes (15). Central memory lymphocytes are defined by expression of CCR7 chemokine receptor like naive T cells but also present memory T cell properties associated with antigen experience. Upon secondary antigen stimulation in lymph nodes central memory cells interact with dendritic cells and can robustly proliferate and differentiate into effector memory cells. Compared to central memory cells, effector memory cells express higher levels of β1 and β2 integrins and receptors for inflammatory chemokines, such as CCR1, CCR3 and CCR5 (15). This re-profiling of surface receptors is responsible for the trafficking of effector memory cells into non-lymphoid sites where they may be able to re-express CCR7 during repeated stimulation (16, 17).

Lymphocyte homing to vessel wall is dependent on chemokines, their receptors and adhesion molecules. Homeostatic chemokines CCL19 (*chemokine C-C motif ligand 19*) and CCL21 (*chemokine C-C motif ligand 21*) which are CCR7 ligands are constitutively expressed and involved in regulation of lymphocyte migration and trafficking but may also promote inflammatory responses (18). Traditionally constitutive chemokines were thought to be responsible for regulating the trafficking of naive T cells to lymphoid organs, whereas inflammatory chemokines were considered as putative mediators of effector memory cells accumulation in inflamed tissues. Further advanced studies have shown, that in chronic inflammation both constitutive and inflammatory chemokines are expressed, thereby sustaining inflammatory activation (19). Therefore CCR7-CCL19/CCL21 pathway might contribute to the pathogenesis or progression of autoimmune diseases such as rheumatoid arthritis (20). According to this concept CCR7-positive cells would accumulate in either activated endothelium or synovium/entheses, with subsequent contribution of CCR7 signaling to Th_1 differentiation and the production of proinflammatory cytokines (21). Based on the ability of CCR7-positive cells to accumulate in both lymphoid and non-lymphoid tissues, CCR7 signaling may also be associated with atherosclerosis. CCL19 and CCL21 are expressed on endothelial cells (4) and perivascular naive T cells are found frequently in patients with rheumatoid arthritis. CCL21 expression by vessel wall may be important in the formation of tertiary lymphoid tissue and the consequent recruitment of $CCR7^+$ lymphocytes (22). Although CCR7 and its ligands CCL19 and CCL21 are present in human atherosclerotic lesions, studies in different experiment mouse models and human studies have brought inconsistent results. Patients with stable and unstable angina have significantly reduced mRNA levels of CCR7 in peripheral blood mononuclear cells compared with healthy controls, and particularly low levels were observed in unstable angina (23). CCL21 and CCR7 mRNA levels were significantly decreased in human atherosclerotic plaques *versus* non-atherosclerotic controls and ox-LDL induced an in vitro downregulation of CCR7 and CCL21 in patients who underwent carotid endarterectomy (24). A study from Cai *et al*. indicated that CCL19/CCL21/CCR7 is expressed in human coronary artery endothelial cells and that monocyte adherence to endothelial cells is CCL19/CCR7 dependant (25). We have previously demonstrated, that classical monocytes ($CD14^{++}CD16^-$) were elevated in AS patients in comparison with healthy controls (26). In *in vitro* models monocyte adherence to endothelial cells was CCL19/CCR7- dependent (25). A common CCR7 dependence of monocyte and lymphocyte migration may suggest monocyte involvement in the modulation of T cell phenotype in AS.

The analysis of peripheral T cells from patients with AS and controls in our study suggested significant differences in CCR7 expression when the two groups were compared. The elevation in circulating T lymphocytes expressing CCR7 in AS (attributable to increased naive $CD8^+$ lymphocytes) may implicate the increased migratory capability of T cells, which may be responsible for the maintenance of the immune response. Increased CCR7 expressing T cells might hypothetically be a compensatory mechanism associated with either enhanced proliferation and survival or redistribution of naive T cells in response to chronic inflammation and endothelial activation. It is to be underlined that the principal difference in T cell subsets between AS patients and controls was found for $CD8^+$ T cells. Admittedly, studies on the role of $CD8^+$ T cells in atherosclerosis are scarce and inconsistent. Some authors have found that they are proatherogenic and can promote development of atherosclerotic plaques (27), while others have suggested their atheroprotective role (28).

Finally, bearing in mind the ability of endothelial cells to present antigens to T cell receptors of $CD8^+$ cells in a MHC (major histocompatibility complex) class I-dependent manner, these pathways could contribute to maintaining of inflammatory activity in AS. HLA-B27 is encoded by an allele of the major MHC class I. One of several proposed mechanisms by which HLA-B27 could play a role in the pathogenesis of AS is the autoreactivity of cytotoxic T lymphocytes in the presence of a MHC class I antigen complex, although the role of molecular mimicry in AS remains uncertain.

Some limitations of the study should be acknowledged. First, due to a rather small sample size of patients the results need to be confirmed in larger studies; second, the possible influence of nonsteroidal anti-inflammatory drugs on endothelial and lymphocyte function should be taken into consideration. Third, the proportion of women was, al-

beit insignificantly, almost 3-fold higher in the control group. In the context of elevated CCR7-positive T lymphocytes in AS patients, serum chemokines CCL19 and CCL21 in AS may be of interest, regrettably they were not assessed in the study. Finally, frequencies of circulating peripheral lymphocytes do not reflect automatically their total pool, because T cell trafficking into inflamed tissues may have influenced the measured proportion of T cell subsets.

In conclusion, to the best of our knowledge, this is the first report showing an increased percentage of T lymphocytes expressing CCR7 chemokine receptor which is accompanied by elevated ADMA in ankylosing spondylitis patients. Whether this changes have a contributory role in the maintenance of chronic inflammatory activation or accelerated atherogenesis in AS remains to be investigated. The lack of direct associations between ADMA and inflammation may suggest presence of other pathogenic mechanisms contributing to accelerated atherogenesis and increased CVD risk in AS.

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