

# Blood Monocyte Heterogeneity and Markers of Endothelial Activation in Ankylosing Spondylitis

Andrzej Surdacki, Joanna Sulicka, Mariusz Korkosz, Tomasz Mikołajczyk, Dorota Telesińska-Jasiówka, Ewa Klimek, Izabella Kierzkowska, Tomasz Guzik, and Tomasz K. Grodzicki

**ABSTRACT. Objective.** Ankylosing spondylitis (AS) is associated with excessive cardiovascular (CV) morbidity. Interactions between activated endothelium and monocytes precede atherosclerotic plaques. Our aim was to quantify blood monocyte subsets in relation to endothelial activation and inflammatory activity in subjects with AS who were free of clinical atherosclerotic CV disease.

**Methods.** Markers of inflammation and endothelial activation were measured in 47 patients with AS receiving no disease-modifying antirheumatic drugs, and 22 healthy controls. Exclusion criteria included atherosclerotic CV disease and traditional risk factors. Flow cytometry was used to identify monocyte subsets: classical CD14<sup>++</sup>CD16<sup>-</sup>, intermediate CD14<sup>++</sup>CD16<sup>+</sup>, and nonclassical CD14<sup>+</sup>CD16<sup>++</sup> monocytes and to evaluate their expression of CD11b and CD11c.

**Results.** Traditional risk factors were comparable among the groups, except for lower high-density lipoprotein cholesterol in AS ( $p = 0.007$ ). Relative to controls, in subjects with AS counts of classical monocytes were higher ( $84.3 \pm 5.4$  vs  $78.9 \pm 5.3\%$  of blood monocytes,  $p < 0.001$ ) and nonclassical monocytes lower ( $2.9 \pm 2.2$  vs  $5.5 \pm 2.3\%$ ,  $p < 0.001$ ). In AS we observed increased soluble intercellular adhesion molecule-1 [251 (224–293) vs 202 (187–230) ng/ml,  $p = 0.002$ ], an endothelial ligand for monocytic  $\beta_2$ -integrin CD11b/CD18. CD11b expression on all 3 monocyte subsets was elevated in 21 AS subjects with a Bath Ankylosing Spondylitis Disease Activity Index score  $\geq 4$  versus the remaining patients ( $p = 0.005$ – $0.03$ ). C-reactive protein, interleukin 6 (IL-6), and pentraxin-3 were increased in AS, in contrast to tumor necrosis factor- $\alpha$  and IL-18. IL-6 correlated with classical monocytes numbers in AS ( $r = 0.56$ ,  $p < 0.0001$ ) but not in the controls ( $r = 0.10$ ,  $p = 0.65$ ).

**Conclusion.** Our findings suggest a contribution of immune dysregulation to enhanced monocyte-endothelial interactions in AS, especially in patients with active disease, which possibly can accelerate atherogenesis on a longterm basis. (First Release Feb 1 2014; J Rheumatol 2014;41:481–9; doi:10.3899/jrheum.130803)

## Key Indexing Terms:

ANKYLOSING SPONDYLITIS

MONOCYTES

ENDOTHELIUM

Rheumatoid arthritis (RA), ankylosing spondylitis (AS), and psoriatic arthritis are associated with increased cardiovascular (CV) mortality and morbidity because of accelerated atherogenesis and disease-specific CV manifestations<sup>1,2,3</sup>. Features of endothelial dysfunction, a predecessor of atherosclerotic plaques, have been observed in patients with each of these inflammatory arthropathies and free of

clinical atherosclerotic CV disease or classical risk factors<sup>4,5,6,7,8,9</sup>.

Interactions between activated endothelium and blood monocytes precede development of atherosclerotic plaques<sup>10</sup>. It has been recognized that not only endothelial dysfunction<sup>10,11,12</sup> but also a shift in blood monocyte subsets can predict adverse CV outcome in various clinical

From the Second Department of Cardiology, the Department of Rheumatology and Balneology, the Division of Rheumatology, Department of Internal Medicine and Gerontology, and the Department of Internal and Agricultural Medicine, Jagiellonian University Medical College and University Hospital; and J. Dietl Hospital, Krakow, Poland.

Supported in part by a research grant from the Ministry of Science and Higher Education, Warsaw, Poland (No. N402 267636).

A. Surdacki, MD, PhD, Professor, Second Department of Cardiology; J. Sulicka, MD, PhD, Physician-researcher, Department of Rheumatology and Balneology; M. Korkosz, MD, PhD, Division of Rheumatology, Department of Internal Medicine and Gerontology, Jagiellonian University Medical College and University Hospital; T. Mikołajczyk, Department of Internal and Agricultural Medicine, Jagiellonian University Medical College and J. Dietl Hospital; D. Telesińska-Jasiówka, MD, University Hospital; E. Klimek, MD, Physician-researcher;

I. Kierzkowska, MD, Physician-researcher, Department of Internal Medicine and Gerontology, Jagiellonian University Medical College and University Hospital; T. Guzik, MD, PhD, Professor, Department of Internal and Agricultural Medicine, Jagiellonian University Medical College and J. Dietl Hospital; T.K. Grodzicki, MD, PhD, Professor, Department of Internal Medicine and Gerontology, Jagiellonian University Medical College and University Hospital.

Dr. Surdacki and Dr. Sulicka contributed equally to this work and are co-first authors.

Address correspondence to Dr. A. Surdacki, 2nd Department of Cardiology, Jagiellonian University Medical College and University Hospital, 17 Kopernika, 31-501 Krakow, Poland.

E-mail: surdacki.andreas@gmx.net.

Accepted for publication November 27, 2013.

settings<sup>13,14,15,16</sup>. Elevated counts of intermediate CD14<sup>++</sup>CD16<sup>+</sup> monocytes (a minor monocyte subpopulation presumably representing a transitory stage of differentiation of predominant classical CD14<sup>++</sup>CD16<sup>-</sup> monocytes toward nonclassical CD14<sup>+</sup>CD16<sup>++</sup> macrophage-like monocytes<sup>17</sup>) were associated with increased risk of ischemic CV events in endstage renal disease<sup>13</sup> and nondialysis chronic kidney disease (CKD)<sup>14</sup>, and excessive risk of major CV events in patients referred for coronary angiography<sup>16</sup>. An increased frequency of CD14/CD16–double-positive monocytes or intermediate CD14<sup>++</sup>CD16<sup>+</sup> monocytes was also reported in RA<sup>18,19,20</sup> and psoriatic arthritis<sup>21</sup>. We have recently described elevated counts of intermediate monocytes in young adult survivors of childhood acute lymphoblastic leukemia<sup>22</sup>, also known to exhibit endothelial dysfunction<sup>23</sup> and to be prone to late CV morbidity<sup>24</sup>.

To our knowledge, no study has focused on blood monocyte heterogeneity in AS to date. Thus, our aim was to estimate frequencies of circulating monocyte subsets and their relations with markers of endothelial dysfunction and inflammatory activity in patients naive to disease-modifying antirheumatic drugs (DMARD) and having AS free of clinical atherosclerotic CV disease.

## MATERIALS AND METHODS

**Subjects.** We studied 47 patients aged 20–47 years (mean  $\pm$  SD,  $33 \pm 6$  yrs; 41 men and 6 women) with AS diagnosed according to the modified New York criteria<sup>25</sup>. All patients had predominantly axial disease and were HLA-B27–positive. The patients were receiving only nonsteroidal anti-inflammatory drugs and none of them had been treated with nonbiologic DMARD, anti-tumor necrosis factor (anti-TNF) agents, or steroids. The mean disease duration was  $8 \pm 5$  years. Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) scores<sup>26</sup> averaged  $4.1 \pm 2.0$  (range: 1.4–9.5). We also recruited a control group of 22 healthy volunteers aged 22–42 years (mean  $30 \pm 7$  yrs; 12 men and 10 women).

Exclusion criteria were common for both groups and included clinical evidence of atherosclerotic CV disease, diabetes, hypertension, an estimated glomerular filtration rate (eGFR) below 90 ml/min per 1.73 m<sup>2</sup> of body surface area, a history of malignancy, acute infections within previous 4 weeks, and any other chronic coexistent diseases. In agreement with the Declaration of Helsinki, the protocol had been approved by the ethics committee of our university, and written informed consent was obtained from each subject before inclusion in the study.

**Study protocol.** The procedure was carried out in the morning in the out-patient clinic of our center. The subjects had been asked to refrain from eating, smoking, and alcohol or caffeine consumption for at least 12 h prior to the study. The personnel involved in the protocol recorded demographic and clinical characteristics, anthropometric measurements were taken, and blood pressure was measured on the left arm after 5 min of rest in a sitting position. Then all participants underwent blood sampling for biochemical assays and flow cytometry.

**Biochemical assays.** A sample of blood was drawn from a left antecubital vein for measurements of serum concentrations of high-sensitivity C-reactive protein (hs-CRP) by immunonephelometry (Dade Behring II Nephelometer, GMI Inc.). Glucose, lipids, and creatinine were measured with a Hitachi 917 analyzer (Roche Diagnostics) using standardized laboratory techniques. An eGFR was calculated by the Chronic Kidney Disease Epidemiology Collaboration formula, which has proven more

accurate than the Modification of Diet in Renal Disease study equation, especially at normal renal function<sup>27</sup>.

Plasma for extended biochemical analyses was collected from EDTA-anticoagulated blood and frozen at  $-80^{\circ}\text{C}$  until assayed. Commercially available ELISA were used to measure plasma levels of TNF- $\alpha$  and interleukin 6 (IL)-6 (Quantikine high-sensitivity kits), monocyte chemotactic protein-1 (MCP-1), pentraxin-3 (PTX-3), soluble forms of vascular cell adhesion molecule-1 (sVCAM-1) and intercellular adhesion molecule-1 (sICAM-1; all R&D Systems), and IL-18 (MBL).

**Flow cytometry.** As described<sup>22</sup>, 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 (Lymphocyte Separation Medium, PAA Laboratories GmbH). One hundred thousand cells were stained for 20 min with fluorochrome-conjugated monoclonal antibodies (anti-CD14-APC-H7, anti-CD16-PE, anti-HLA-DR-PE-Cy7, anti-CD11b-Pacific Blue; and anti-CD11c-APC; Becton Dickinson Biosciences — Pharmingen) and then washed with phosphate buffered saline containing 1% fetal bovine serum (GIBCO). The cells were processed in the FACSCanto II flow cytometer (BD Biosciences) and then analyzed with FACSDiva software (BD Biosciences) and FlowJo (TreeStar Inc.). The cells were gated in a side scatter/forward scatter plot with the scatter gate for monocytes partially extending into lymphocytes<sup>28</sup>. The cells containing all monocytes and a part of the lymphocyte population were then gated in an HLA-DR/CD14 plot to exclude HLA-DR–negative natural killer cells (which would otherwise contaminate the CD14<sup>+</sup>CD16<sup>++</sup> subpopulation) and finally analyzed for CD14 and CD16 expression<sup>28,29</sup>. Monocyte subsets were defined according to the expression of CD16 (Fc $\gamma$  receptor type III) and CD14 (lipopolysaccharide receptor) as classical monocytes (CD14<sup>++</sup>CD16<sup>-</sup>), intermediate monocytes (CD14<sup>++</sup>CD16<sup>+</sup>), and nonclassical monocytes (CD14<sup>+</sup>CD16<sup>++</sup>)<sup>17</sup>. The expression of CD11b and CD11c on the 3 monocyte subsets was quantified. The results have been presented as either the percentage of cells expressing individual markers, absolute cell counts (per  $\mu\text{l}$  of blood), or mean fluorescence intensity (MFI). Total numbers of monocytes were calculated using peripheral white blood cell counts. In separate experiments BD Trucount beads were used, confirming that both methods yielded similar absolute values of cell counts.

All experiments were performed under standardized 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. Simultaneously, in each case we checked the proper operation of the flow cytometer using BD Cytometer Setup and Tracking beads. Also, the operational stability was verified, so that the individual measurements did not differ significantly one from the other. Flow cytometry compensation was verified regularly using cells studied in the experiment and CompBeads. One operator performed the sample collection and the data analysis.

**Statistical analysis.** Data are presented as mean  $\pm$  SD unless stated otherwise or medians (interquartile range) 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 variance (homoscedasticity) by Levene's test. Intergroup differences were assessed by the unpaired 2-sided Student's *t* test for continuous variables and Fisher's exact test for proportions. Logarithmic transformation (ln, natural logarithm) was applied, when necessary, to obtain a normal distribution (hs-CRP, TNF- $\alpha$ , IL-6, sVCAM-1, PTX-3, percentage of intermediate monocytes) or to eliminate the nonuniformity of variances. The 2-sided Welch's *t* test was used in case of the lack of homogeneity of variances even in ln-transformed data. A *p*-value below 0.05 was considered significant. Bivariate relations between continuous variables were estimated by Pearson's correlation coefficients (*r*). Owing to multiple testing, exclusive correlations with  $p \leq 0.001$  were inferred as significant. Analyses were performed by means of the Statistica data analysis software system (version 10.0.1011.0; StatSoft Inc.).

**Table 1.** Clinical and conventional biochemical characteristics of patients with ankylosing spondylitis (AS) and control subjects. Data are shown as medians (interquartile range) or n (%). P values in boldface are statistically significant.

Characteristic	Patients with AS, n = 47	Controls, n = 22	p
Age, yrs	33 (28–36)	29 (22–38)	0.10
BASDAI	3.7 (2.6–4.7)	NA	NA
Disease duration, yrs	6 (4–10)	NA	NA
Male sex	41 (87)	13 (59)	<b>0.008</b>
Body mass index, kg/m <sup>2</sup>	24.3 (22.5–26.6)	23.3 (22.6–24.6)	0.15
Smoking	14 (30)	6 (27)	0.83
Mean blood pressure, mmHg	92 (87–97)	90 (87–92)	0.13
Glucose, mmol/l	4.7 (4.5–5.0)	4.7 (4.4–4.9)	0.72
eGFR, ml/min per 1.73 m <sup>2</sup>	121 (110–132)	120 (99–141)	0.15
Total cholesterol, mmol/l	5.0 (4.2–5.8)	4.8 (4.5–5.6)	0.66
LDL cholesterol, mmol/l	3.1 (2.4–3.8)	2.8 (2.3–3.5)	0.11
HDL cholesterol, mmol/l	1.3 (1.2–1.5)	1.7 (1.4–2.0)	<b>0.007*</b>
Total/HDL cholesterol	3.7 (3.2–4.3)	3.1 (2.5–3.7)	<b>0.01</b>
Triglycerides, mmol/l	0.9 (0.6–1.2)	0.9 (0.7–1.2)	0.48
hs-CRP, mg/l	5.05 (2.57–16.50)	0.88 (0.39–1.48)	<b>&lt; 0.001<sup>†</sup></b>
Fibrinogen, g/l	4.17 (3.40–5.23)	3.09 (2.30–3.40)	<b>&lt; 0.001*</b>

\* By Welch's t test for untransformed data because of the non-uniformity of variances. <sup>†</sup> By Welch's t test for ln-transformed data because of the lack of normal distribution and the non-uniformity of variances. BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; NA: not applicable; eGFR: estimated glomerular filtration rate; LDL: low-density lipoprotein; HDL: high-density lipoprotein; hs-CRP: high-sensitivity C-reactive protein.

## RESULTS

**Clinical and biochemical characteristics of the patients with AS and healthy controls.** Traditional CV risk factors were similar in patients with AS and healthy controls except for lower high-density lipoprotein (HDL) cholesterol, in agreement with a previous report<sup>30</sup>, and a higher ratio of total cholesterol to HDL cholesterol in AS (Table 1). Additionally, levels of fibrinogen, hs-CRP, IL-6, sICAM-1, and PTX-3 were significantly increased in patients with AS, whereas TNF- $\alpha$ , sVCAM-1, IL-18, and MCP-1 were similar between the groups (Table 2).

**Blood monocyte subsets and their expression of CD11b and CD11c in patients with AS and healthy controls.** Compared to the controls, patients with AS exhibited significantly

higher counts of classical CD14<sup>++</sup>CD16<sup>-</sup> monocytes and lower numbers of nonclassical CD14<sup>+</sup>CD16<sup>++</sup> monocytes (Table 3). There were no differences in the expression of CD11b and CD11c on all 3 monocyte subsets between AS subjects and the controls ( $p > 0.1$ ).

Pooling both groups as a whole, men tended to have higher total monocyte counts than women ( $536 \pm 208$  vs  $436 \pm 115$  cells/ $\mu$ l,  $p = 0.09$ ). Because the percentage of men was higher in patients with AS (87% vs 55%), we repeated the comparisons after limitation of the analysis to male subjects, which revealed similar directions and levels of significance for intergroup differences in classical ( $p = 0.001$ ) and nonclassical ( $p < 0.001$ ) monocytes.

There was a correlation between IL-6 and the count of

**Table 2.** Extended inflammatory markers in ankylosing spondylitis (AS) patients and controls. Data are shown as medians (interquartile range). P values in boldface are statistically significant.

Characteristic	Patients with AS, n = 47	Controls, n = 22	p
TNF- $\alpha$ , pg/ml	1.76 (1.57–2.13)	1.49 (1.34–1.96)	0.17*
IL-6, pg/ml	3.51 (1.79–6.19)	0.74 (0.56–1.15)	<b>&lt; 0.001<sup>†</sup></b>
IL-18, pg/ml	407 (322–493)	417 (285–472)	0.50
sICAM-1, ng/ml	251 (224–293)	202 (187–230)	<b>0.002</b>
sVCAM-1, ng/ml	585 (496–644)	551 (507–635)	0.69*
MCP-1, pg/ml	256 (211–307)	256 (218–293)	0.62
Pentraxin-3, pg/ml	589 (483–772)	420 (364–483)	<b>0.002*</b>

\* By Student's t test for natural logarithm (ln)-transformed data to obtain a normal distribution. <sup>†</sup> By Welch's t test for ln-transformed data because of the lack of normal distribution and the nonuniformity of variances. TNF- $\alpha$ : tumor necrosis factor- $\alpha$ ; IL: interleukin; sICAM-1: soluble intercellular adhesion molecule-1; sVCAM-1: soluble vascular cell adhesion molecule-1; MCP-1: monocyte chemoattractant protein-1.

Table 3. Monocyte subsets and total monocyte counts in ankylosing spondylitis (AS) patients and healthy subjects. Data are shown as mean  $\pm$  SD. P values in boldface are statistically significant.

Subset of Blood Monocytes	Patients with AS, n = 47	Healthy Subjects, n = 22	p
CD14 <sup>++</sup> CD16 <sup>-</sup> classical monocytes			
% of monocytes	84.3 $\pm$ 5.4	78.9 $\pm$ 5.3	<b>&lt; 0.001</b>
Per $\mu$ l of blood	469 $\pm$ 194	364 $\pm$ 124	<b>0.02</b>
CD14 <sup>++</sup> CD16 <sup>+</sup> intermediate monocytes			
% of monocytes	5.5 $\pm$ 3.3	5.0 $\pm$ 1.9	0.60*
Per $\mu$ l of blood	28 $\pm$ 15	23 $\pm$ 11	0.21
CD14 <sup>+</sup> CD16 <sup>++</sup> nonclassical monocytes			
% of monocytes	2.9 $\pm$ 2.2	5.5 $\pm$ 2.3	<b>&lt; 0.001</b>
Per $\mu$ l of blood	15 $\pm$ 10	25 $\pm$ 12	<b>&lt; 0.001</b>
Total monocytes (per $\mu$ l of blood)	542 $\pm$ 211	462 $\pm$ 148	0.12

\* By Student's t test for natural logarithm-transformed data to get a normal distribution.

Table 4. Pearson's correlation coefficients between inflammatory markers, disease activity, monocyte subsets size, and monocytic CD11b and CD11c expression in patients with ankylosing spondylitis. Data in boldface are statistically significant.

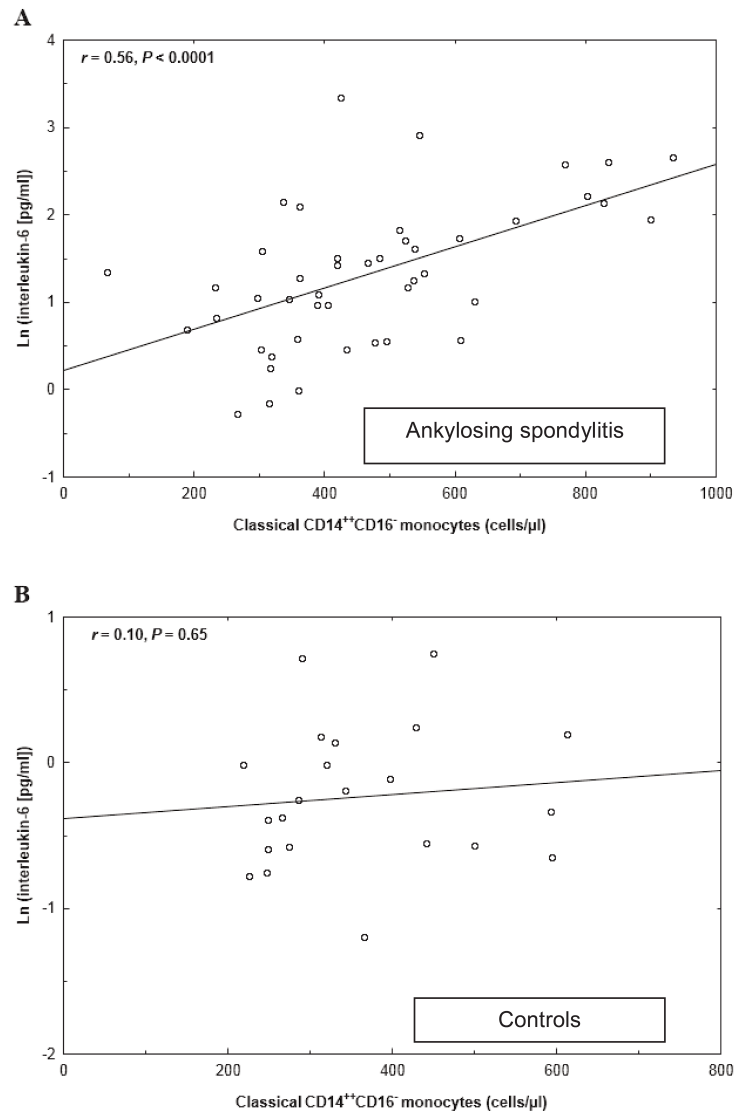
	hs-CRP	Fibr.	IL-6	IL-18	TNF- $\alpha$	BASDAI
sICAM-1	0.15	0.30	0.08	0.22	-0.04	0.11
sVCAM-1	-0.11	-0.19	-0.21	-0.02	0.06	-0.16
Pentraxin-3	-0.04	-0.08	0.17	0.07	-0.19	0.23
Classical monocytes (%)	0.33	0.37	<b>0.58**</b>	0.05	0.00	0.03
Classical monocytes, per $\mu$ l of blood	0.39	0.42	<b>0.56**</b>	-0.04	0.03	0.05
CD11b expression on classical monocytes	0.19	0.30	0.26	0.04	-0.12	<b>0.47*</b>
CD11c expression on classical monocytes	-0.21	-0.19	-0.02	-0.03	0.04	0.02
Intermediate monocytes (%)	0.08	-0.06	0.02	-0.02	0.08	0.11
Intermediate monocytes, per $\mu$ l of blood	0.27	0.28	0.35	0.13	0.10	0.28
CD11b expression on intermediate monocytes	0.20	0.33	0.27	0.04	-0.15	<b>0.49*</b>
CD11c expression on intermediate monocytes	-0.06	-0.06	-0.02	0.09	0.02	0.03
Nonclassical monocytes (%)	-0.19	-0.18	<b>-0.53*</b>	-0.07	-0.01	-0.02
Nonclassical monocytes, per $\mu$ l of blood	-0.13	-0.04	-0.32	0.01	0.02	0.13
CD11b expression on nonclassical monocytes	0.10	0.23	0.13	0.04	-0.19	<b>0.52*</b>
CD11c expression on nonclassical monocytes	-0.09	-0.11	0.08	0.05	0.10	-0.03

Levels of hs-CRP, IL-6, TNF- $\alpha$ , sVCAM-1, PTX-1, and percentage of intermediate monocytes were log-transformed prior to analysis. Because of multiple comparisons, exclusively p values  $\leq$  0.001 were inferred significant. \* p  $\leq$  0.001, \*\* p < 0.0001. Fibr: fibrinogen; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; hs-CRP: high-sensitivity C-reactive protein; TNF- $\alpha$ : tumor necrosis factor- $\alpha$ ; IL: interleukin; sICAM-1: soluble intercellular adhesion molecule-1; sVCAM-1: soluble vascular cell adhesion molecule-1.

classical monocytes in patients with AS ( $r = 0.56$ ,  $p < 0.0001$ ; (Table 4 and Figure 1A) but not in the controls ( $r = 0.10$ ,  $p = 0.65$ ; Figure 1B). Neither HDL cholesterol nor the ratio of total to HDL cholesterol correlated to any of the variables derived from flow cytometry ( $p \geq 0.1$ ).

*Clinical and biochemical characteristics, monocyte subset size, and monocytic expression of CD11b and CD11c in relation to disease activity.* BASDAI correlated to the expression of CD11b on classical ( $r = 0.47$ ,  $p = 0.001$ ), intermediate ( $r = 0.49$ ,  $p < 0.001$ ), and nonclassical monocytes ( $r = 0.52$ ,  $p < 0.001$ ; Table 4). Accordingly, compared to AS patients with a BASDAI score < 4 ( $n = 26$ ), AS patients with BASDAI  $\geq 4$  ( $n = 21$ ) had higher monocytic expression of CD11b on classical ( $1529 \pm 1006$  vs  $998 \pm 591$  MFI,  $p =$

$0.03$ ; Figure 2A), intermediate ( $1591 \pm 899$  vs  $1009 \pm 579$  MFI,  $p = 0.01$ ; Figure 2B), and nonclassical monocytes ( $607 \pm 185$  vs  $461 \pm 143$  MFI,  $p = 0.005$ ; Figure 2C). Relative increases in CD11b expression on classical, intermediate, and nonclassical monocytes associated with a high disease activity averaged 53%, 58%, and 32%, respectively, with the reference to the remaining subjects with AS. The expression of CD11b was reduced by about one-third in the patients with AS who had a low disease activity, relative to controls on all 3 monocyte subpopulations: classical ( $998 \pm 591$  vs  $1539 \pm 629$  MFI,  $p = 0.003$ ), intermediate ( $1009 \pm 579$  vs  $1523 \pm 584$  MFI,  $p = 0.004$ ), and nonclassical monocytes ( $461 \pm 143$  vs  $729 \pm 566$  MFI,  $p = 0.02$ ).



**Figure 1.** A positive correlation between the numbers of classical monocytes and interleukin 6 levels in patients with ankylosing spondylitis (A) but not in the controls (B). Levels of interleukin 6 were transformed in natural logarithm (Ln) to obtain a normal distribution.

The AS patients with a BASDAI score  $\geq 4$  and  $< 4$  did not differ in terms of traditional risk factors ( $p > 0.1$ ), levels of sICAM-1 ( $270 \pm 70$  vs  $262 \pm 50$  ng/ml,  $p = 0.7$ , respectively), sVCAM-1 [ $541$  ( $491$ – $607$ ) vs  $591$  ( $500$ – $658$ ) ng/ml,  $p = 0.08$ ], hs-CRP [ $7.02$  ( $2.57$ – $18.20$ ) vs  $5.05$  ( $2.95$ – $13.60$ ) mg/l,  $p = 0.8$ ], PTX-3 [ $631$  ( $421$ – $739$ ) vs  $551$  ( $483$ – $772$ ) pg/ml,  $p = 0.6$ ], TNF- $\alpha$  [ $1.77$  ( $1.50$ – $2.20$ ) vs  $1.80$  ( $1.58$ – $2.07$ ) pg/ml,  $p = 0.8$ ], IL-6 [ $3.09$  ( $2.24$ – $6.98$ ) vs  $3.55$  ( $1.80$ – $5.55$ ) pg/ml,  $p = 0.5$ ], IL-18 [ $446 \pm 137$  vs  $422 \pm 153$  pg/ml,  $p = 0.6$ ], MCP-1 [ $252 \pm 72$  vs  $264 \pm 74$  pg/ml,  $p = 0.6$ ], and size of monocyte subsets ( $p = 0.4$ – $0.8$ ; Table 4).

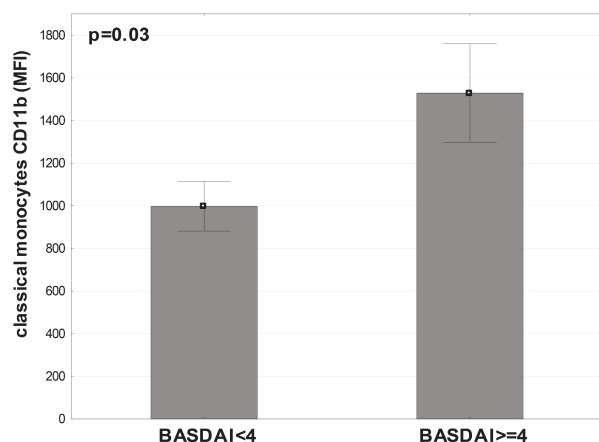
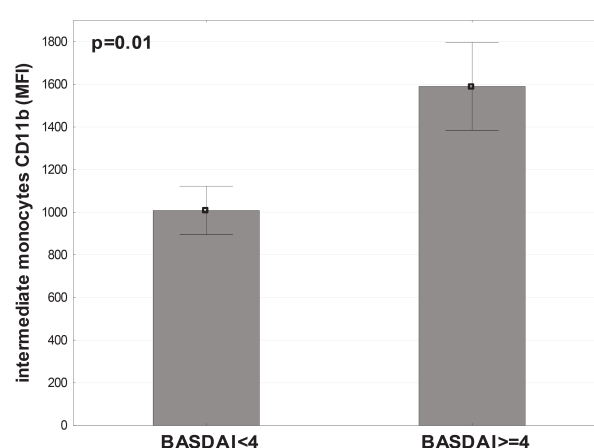
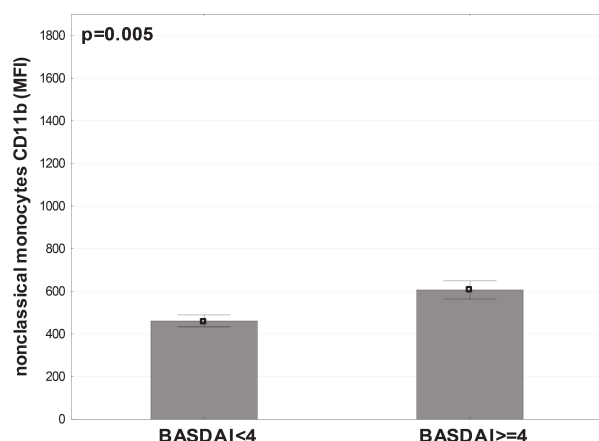
The expression of CD11c was similar in patients with AS who had high disease activity, low disease activity, and the controls on classical ( $1475 \pm 638$  vs  $1522 \pm 497$  vs  $1695 \pm$

$1231$  MFI, respectively), intermediate ( $3381 \pm 1025$  vs  $3468 \pm 1040$  vs  $3983 \pm 2612$  MFI), and nonclassical monocytes ( $3620 \pm 972$  vs  $3809 \pm 1029$  vs  $3635 \pm 2359$  MFI; all inter-group  $p = 0.3$ – $0.9$ ).

## DISCUSSION

Our salient finding was a higher number of classical CD14<sup>++</sup>CD16<sup>-</sup> blood monocytes and its association with elevated IL-6 levels in DMARD-naïve patients with AS. This study is the first, to our knowledge, to report a shift in blood monocyte subpopulations in AS. Additionally, in AS we observed increased sICAM-1, a marker of endothelial activation and an endothelial ligand for monocytic  $\beta_2$ -integrin CD11b/CD18. Moreover, on all 3 monocyte subsets the expression of CD11b was decreased in AS



**A****B****C**

**Figure 2.** Elevated CD11b expression on classical (A), intermediate (B), and nonclassical (C) monocytes in subjects with ankylosing spondylitis and a BASDAI score  $\geq 4$  ( $n = 21$ ) compared to the remaining patients ( $n = 26$ ). Data are shown as means  $\pm$  SE of the mean; p values by Student's t test. BASDAI: Bath Ankylosing Spondylitis Disease Activity Index score; MFI: mean fluorescence intensity.

subjects with low disease activity compared to those with high disease activity, or healthy controls.

The shift in monocyte subsets in AS differed from the previously observed expansion of CD14/CD16–double positive monocytes<sup>18,20</sup> or CD14<sup>++</sup>CD16<sup>+</sup> intermediate monocytes<sup>19</sup> in RA. This might result from distinct pathogenesis of these 2 forms of inflammatory arthritis. However, we have not studied patients with RA, which limits comparisons of our findings with those reports<sup>18,19,20</sup>.

That the observed increase in CD14<sup>++</sup>CD16<sup>–</sup> monocyte counts in AS correlated to 6-fold elevated IL-6 levels, but not TNF- $\alpha$  or IL-18 concentrations, is compatible with a high secretion of IL-6 by classical monocytes from healthy individuals in response to lipopolysaccharide<sup>31,32</sup>. Because IL-6 (besides inducing the synthesis of acute-phase reactants) plays a role in the differentiation of Th17<sup>33</sup> and Th2 cells<sup>34</sup>, this relationship may reflect the contribution of

a shift in blood monocyte subpopulations to the pathogenesis of AS, reportedly associated with a 10-fold increase in the percentage of Th17 cells among peripheral blood mononuclear cells without *in vitro* stimulation<sup>35</sup>. Nevertheless, it is unknown whether classical monocytes have the same properties in patients with AS and healthy subjects.

Previous reports relating monocyte heterogeneity to CV prognosis are inconsistent. A higher frequency of intermediate monocytes but not classical monocytes independently predicted adverse CV outcome in 94 patients receiving dialysis and in 119 nondialysis CKD subjects followed for about 3 and 5 years, respectively<sup>13,14</sup>. In 951 patients referred for elective coronary angiography, mainly with prevalent coronary artery disease (CAD) or cerebrovascular disease, both classical and intermediate monocytes emerged as univariate predictors of major CV events during a mean followup of 2.6 years, whereas upon multivariate

adjustment the statistical significance was retained exclusively for intermediate monocytes<sup>16</sup>. Therefore, our findings might suggest that excessive CV risk in AS might be independent of a shift in favor of classical monocytes.

On the other hand, classical monocytes, but not other monocyte subsets, independently predicted ischemic CV events in 700 randomly selected participants of the cardiovascular arm of the Malmö Diet and Cancer study over a 15-year followup<sup>15</sup>. Additionally, in a group of high-risk patients with stable CAD<sup>36</sup>, the proportion of classical monocytes was elevated at the expense of nonclassical monocytes, as in our AS subjects, compared to low-risk and medium-risk groups delineated on the basis of the number of traditional risk factors in 80 subjects with CAD. Further, Urra, *et al*<sup>37</sup> found a positive association between classical monocytes (which exhibited the highest platelet-monocyte interactions) and 3-month mortality in 46 consecutive patients with stroke.

However, the published observations on the association between adverse CV outcome and classical CD14<sup>++</sup>CD16<sup>-</sup> monocytes or intermediate CD14<sup>++</sup>CD16<sup>+</sup> monocytes may be not entirely contradictory because Cros, *et al*<sup>31</sup> have provided evidence (by means of whole-genome expression arrays) that both classical and intermediate human monocytes resemble murine Ly6C<sup>high</sup>Gr<sup>+</sup> monocytes, exhibiting preferential homing to activated endothelium and atherosclerotic plaques in experimental models of atherosclerosis<sup>38,39,40</sup>. In addition, preferential transendothelial migration in response to MCP-1 was demonstrated for human classical monocytes as compared to the other monocyte subsets<sup>41</sup>. Moreover, classical monocytes are the main source of reactive oxygen species<sup>31,42</sup> known to impair the activity of nitric oxide<sup>43</sup>, an endogenous antiatherogenic molecule.

Thus, an increased frequency of classical monocytes in our subjects with AS who had elevated sICAM-1, an index of endothelial activation and an endothelial ligand for monocytic  $\beta_2$ -integrin CD11b/CD18<sup>44</sup>, supported by a relative increase in the expression of CD11b on classical and intermediate monocytes by over 50% in patients with active disease compared to those with low disease activity, might reflect enhanced leukocyte-endothelial interactions and chronic inflammation within the vascular wall<sup>10</sup>. Because BASDAI score had been reportedly associated with traditional risk factors, including metabolic syndrome components<sup>45,46</sup>, the leukocyte-endothelial interactions could potentiate pro-atherogenic effects of the classical risk factors in patients with high disease activity. Admittedly, monocytic CD11b expression did not differ between the patients with AS who had active disease and the controls; nevertheless, a decreased CD11b expression on monocytes of the subjects with AS who had low disease activity could hypothetically reflect an effective compensatory antiinflammatory mechanism that was absent in those with a BASDAI

score  $\geq 4$ . This suggestion remains speculative and requires further investigation.

On the other hand, the biological significance of relatively small differences in sICAM-1 levels and the frequency of classical monocytes remains unclear. Nevertheless, compared to controls, patients with AS exhibited higher average relative elevations of these variables [classical monocytes: 469 vs 364 per  $\mu$ l of blood (29%); sICAM-1: 251 vs 202 ng/ml (25%)] than those previously reported in participants who later experienced an ischemic CV event versus the event-free subjects in prospective studies [classical monocytes: 344 vs 297 per  $\mu$ l of blood (16%) in the Malmö Diet and Cancer study<sup>15</sup>; sICAM-1: 289 vs 244 ng/ml (18%) in the Atherosclerosis Risk in Communities study<sup>47</sup>].

It is noteworthy that PTX-3, elevated in patients with AS, is released locally from cells involved in atherogenesis and (despite its atheroprotective effects) can be considered a marker of vascular inflammation<sup>48</sup>. Further, higher PTX-3 levels predicted CV mortality independently of hs-CRP and traditional risk factors in subjects free of prevalent CV disease<sup>49</sup>. However, we have provided no direct evidence supporting the notion of a better ability of PTX-3, adhesion molecules, or monocyte subsets to predict accelerated atherogenesis compared to classical risk factors and conventional inflammatory markers in AS. To verify this speculative concept, further studies are warranted, relating these variables to coexistent atherosclerotic CV disease or subclinical atherosclerosis in patients with AS.

Several limitations of our study need to be acknowledged. First, the small number of subjects constrains the conclusions based on our findings. Second, we have measured cytokines and chemokines in plasma, whereas the assessment of cytokine generation in response to various stimuli and the expression of chemokine receptors by different monocyte subsets would add information to the *in situ* plasma levels<sup>18,19,31,32,42</sup>. Third, we are not able to exclude the influence of nonsteroidal antiinflammatory drugs on the activation of both monocytes and endothelial cells in patients with AS. Fourth, blood monocyte subpopulations might be affected by lipids<sup>50</sup>, and a lower level of HDL cholesterol was observed in subjects with AS. However, no lipid variable correlated to the size of any of the monocyte subsets or their  $\beta_2$ -integrin expression in our study. Finally, although we made every effort to ensure similar characteristics in both groups, the percentage of men was higher in AS. Rogacev, *et al*<sup>16</sup> described higher absolute counts of classical monocytes in men referred for elective coronary angiography mainly because of increased numbers of total monocytes, also present in our data. Nevertheless, we observed intergroup differences in both relative and absolute counts of classical monocytes, which argues against the notion of sex differences in total monocyte counts as an explanation of our results. In addition, the

results have not changed after limitation of the analysis to male subjects.

Patients with AS exhibit a shift in blood monocyte subpopulations. Our preliminary cross-sectional findings suggest contribution of immune dysregulation to enhanced monocyte-endothelial interactions in AS, especially in patients with active disease, which possibly can accelerate atherogenesis on a longterm basis. Further longitudinal studies are warranted to determine the relevance of these abnormalities for late CV morbidity.

## REFERENCES

- Peters MJ, Symmons DP, McCarey D, Dijkmans BA, Nicola P, Kvien TK, et al. EULAR evidence-based recommendations for cardiovascular risk management in patients with rheumatoid arthritis and other forms of inflammatory arthritis. *Ann Rheum Dis* 2010;69:325-31.
- Peters MJ, van der Horst-Bruinsma IE, Dijkmans BA, Nurmohamed MT. Cardiovascular risk profile of patients with spondylarthropathies, particularly ankylosing spondylitis and psoriatic arthritis. *Semin Arthritis Rheum* 2004;34:585-9.
- Szabo SM, Levy AR, Rao SR, Kirbach SE, Lacaille D, Cifaldi M, et al. Increased risk of cardiovascular and cerebrovascular diseases in individuals with ankylosing spondylitis: a population-based study. *Arthritis Rheum* 2011;63:3294-304.
- Vaudo G, Marchesi S, Gerli R, Allegrucci R, Giordano A, Siepi D, et al. Endothelial dysfunction in young patients with rheumatoid arthritis and low disease activity. *Ann Rheum Dis* 2004;63:31-5.
- Surdacki A, Martens-Lobenhoffer J, Wloch A, Marewicz E, Rakowski T, Wieczorek-Surdacka E, et al. Elevated plasma asymmetric dimethyl-L-arginine levels are linked to endothelial progenitor cell depletion and carotid atherosclerosis in rheumatoid arthritis. *Arthritis Rheum* 2007;56:809-19.
- Surdacki A, Martens-Lobenhoffer J, Wloch A, Gluszkop P, Rakowski T, Dubiel JS, et al. Plasma asymmetric dimethylarginine is related to anticitrullinated protein antibodies in rheumatoid arthritis of short duration. *Metabolism* 2009;58:316-8.
- Aari I, Okan T, Akar S, Cece H, Altay C, Secil M, et al. Impaired endothelial function in patients with ankylosing spondylitis. *Rheumatology* 2006;45:283-6.
- Gonzalez-Juanatey C, Llorca J, Amigo-Diaz E, Dierssen T, Martin J, Gonzalez-Gay MA. High prevalence of subclinical atherosclerosis in psoriatic arthritis patients without clinically evident cardiovascular disease or classic atherosclerosis risk factors. *Arthritis Rheum* 2007;57:1074-80.
- Sari I, Kebapcilar L, Alacacioglu A, Bilgi O, Yildiz Y, Taylan A, et al. Increased levels of asymmetric dimethylarginine (ADMA) in patients with ankylosing spondylitis. *Intern Med* 2009;48:1363-8.
- Hansson GK. Mechanisms of disease: inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* 2005;352:1685-95.
- Al Suwaidi J, Hamasaki S, Higano ST, Nishimura RA, Holmes DR Jr, Lerman A. Long-term follow-up of patients with mild coronary artery disease and endothelial dysfunction. *Circulation* 2000;101:948-54.
- Schächinger V, Britten MB, Zeiher AM. Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease. *Circulation* 2000;101:1899-906.
- Heine GH, Ulrich C, Seibert E, Seiler S, Marell J, Reichart B, et al. CD14++CD16+ monocytes but not total monocyte numbers predict cardiovascular events in dialysis patients. *Kidney Int* 2008;73:622-9.
- Rogacev KS, Seiler S, Zawada AM, Reichart B, Herath E, Roth D, et al. CD14++CD16+ monocytes and cardiovascular outcome in patients with chronic kidney disease. *Eur Heart J* 2011;32:84-92.
- Berg KE, Ljungcrantz I, Andersson L, Bryngelsson C, Hedblad B, Fredrikson GN, et al. Elevated CD14++CD16-monocytes predict cardiovascular events. *Circulation Cardiovasc Genet* 2012;5:122-31.
- Rogacev KS, Cremers B, Zawada AM, Seiler S, Binder N, Ege P, et al. CD14++CD16+ monocytes independently predict cardiovascular events: a cohort study of 951 patients referred for elective coronary angiography. *J Am Coll Cardiol* 2012;60:1512-20.
- Ziegler-Heitbrock L, Ancuta P, Crowe S, Dalod M, Grau V, Hart DN, et al. Nomenclature of monocytes and dendritic cells in blood. *Blood* 2010;116:e74-80.
- Kawanaka N, Yamamura M, Aita T, Morita Y, Okamoto A, Kawashima M, et al. CD14+, CD16+ blood monocytes and joint inflammation in rheumatoid arthritis. *Arthritis Rheum* 2002;46:2578-86.
- Rosol M, Kraus S, Pierer M, Baerwald C, Wagner U. The CD14 brightCD16+ monocyte subset is expanded in rheumatoid arthritis and promotes expansion of the Th17-cell population. *Arthritis Rheum* 2012;64:671-7.
- Cooper DL, Martin SG, Robinson JJ, Mackie SL, Charles CJ, Nam J, et al. FcγRIIIa expression on monocytes in rheumatoid arthritis: role in immune-complex stimulated TNF production and non-response to methotrexate therapy. *PLoS ONE* 2012;7:e28918.
- Chiu YG, Shao T, Feng C, Mensah KA, Thullen M, Schwarz EM, et al. CD16 (FcγRIII) as a potential marker of osteoclast precursors in psoriatic arthritis. *Arthritis Res Ther* 2010;12:R14.
- Sulicka J, Surdacki A, Mikołajczyk T, Strach M, Gryglewska B, Ćwiklińska M, et al. Elevated markers of inflammation and endothelial activation and increased counts of intermediate monocytes in adult survivors of childhood acute lymphoblastic leukemia. *Immunobiology* 2013;218:810-6.
- Dengel DR, Ness KK, Glasser SP, Williamson EB, Baker KS, Gurney JG. Endothelial function in young adult survivors of childhood acute lymphoblastic leukemia. *J Pediatr Hematol Oncol* 2008;30:20-5.
- Mulrooney DA, Yeazel MW, Kawashima T, Mertens AC, Mitby P, Stovall M, et al. Cardiac outcomes in a cohort of adult survivors of childhood and adolescent cancer: retrospective analysis of the Childhood Cancer Survivor Study cohort. *BMJ* 2009;339:b4606.
- van der Linden S, Valkenburg HA, Cats A. Evaluation of diagnostic criteria for ankylosing spondylitis. A proposal for modification of the New York criteria. *Arthritis Rheum* 1984;27:361-8.
- Garrett S, Jenkinson T, Kennedy LG, Whitelock H, Gaisford P, Calin A. A new approach to defining disease status in ankylosing spondylitis: the Bath Ankylosing Spondylitis Disease Activity Index. *J Rheumatol* 1994;21:2286-91.
- Levey AS, Stevens LA, Schmid CH, Zhang YL, Castro AF 3rd, Feldman HI, et al; CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration). A new equation to estimate glomerular filtration rate. *Ann Intern Med* 2009;150:604-12.
- Heimbeck I, Hofer TP, Eder C, Wright AK, Frankenberger M, Marei A, et al. Standardized single-platform assay for human monocyte subpopulations: lower CD14+CD16++ monocytes in females. *Cytometry A* 2010;77:823-30.
- Siedlar M, Strach M, Bukowska-Strakova K, Lenart M, Szaflarska A, Węglarczyk K, et al. Preparations of intravenous immunoglobulins diminish the number and proinflammatory response of CD14+CD16++ monocytes in common variable immunodeficiency (CVID) patients. *Clin Immunol* 2011;139:122-32.
- Divecha H, Sattar N, Rumley A, Cherry L, Lowe GD, Sturrock R. Cardiovascular risk parameters in men with ankylosing spondylitis in comparison with non-inflammatory control subjects: relevance of systemic inflammation. *Clin Sci* 2005;109:171-6.



31. Cros J, Cagnard N, Woollard K, Patey N, Zhang SY, Senechal B, et al. Human CD14<sup>dim</sup> monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors. *Immunity* 2010;33:375-86.
32. Ziegler-Heitbrock HW, Ströbel M, Kieper D, Fingerle G, Schlunck T, Petersmann I, et al. Differential expression of cytokines in human blood monocyte subpopulations. *Blood* 1992;79:503-11.
33. Samson M, Audia S, Janikashvili N, Ciudad M, Trad M, Fraszczak J, et al. Brief report: inhibition of interleukin-6 function corrects Th17/Treg cell imbalance in patients with rheumatoid arthritis. *Arthritis Rheum* 2012;64:2499-503.
34. Rincón M, Anguita J, Nakamura T, Fikrig E, Flavell RA. Interleukin (IL)-6 directs the differentiation of IL-4-producing CD4<sup>+</sup> T cells. *J Exp Med* 1997;185:461-9.
35. Limón-Camacho L, Vargas-Rojas MI, Vázquez-Mellado J, Casasola-Vargas J, Moctezuma JF, Burgos-Vargas R, et al. In vivo peripheral blood proinflammatory T cells in patients with ankylosing spondylitis. *J Rheumatol* 2012;39:830-5.
36. Hristov M, Leyendecker T, Schuhmann C, Von Hundelshausen P, Heussen N, Kehmeier E, et al. Circulating monocyte subsets and cardiovascular risk factors in coronary artery disease. *Thromb Haemost* 2010;104:412-4.
37. Urra X, Villamor N, Amaro S, Gómez-Choco M, Obach V, Oleaga L, et al. Monocyte subtypes predict clinical course and prognosis in human stroke. *J Cereb Blood Flow Metab* 2009;29:994-1002.
38. Swirski FK, Weissleder R, Pittet MJ. Heterogeneous in vivo behavior of monocyte subsets in atherosclerosis. *Arterioscler Thromb Vasc Biol* 2009;29:1424-32.
39. An G, Wang H, Tang R, Yago T, McDaniel JM, McGee S, et al. P-selectin glycoprotein ligand-1 is highly expressed on Ly-6Chi monocytes and a major determinant for Ly-6Chi monocyte recruitment to sites of atherosclerosis in mice. *Circulation* 2008;117:3227-37.
40. Swirski FK, Libby P, Aikawa E, Alcaide P, Luscinskas FW, Weissleder R, et al. Ly-6Chi monocytes dominate hypercholesterolemia-associated monocytes and give rise to macrophages in atheromata. *J Clin Invest* 2007;117:195-205.
41. Ancuta P, Rao R, Moses A, Mehle A, Shaw SK, Luscinskas FW, et al. Fractalkine preferentially mediates arrest and migration of CD16<sup>+</sup> monocytes. *J Exp Med* 2003;197:1701-7.
42. Grage-Griebenow E, Flad HD, Ernst M. Heterogeneity of human peripheral blood monocyte subsets. *J Leukoc Biol* 2001;69:11-20.
43. Gryglewski RJ, Palmer RM, Moncada S. Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. *Nature* 1986;320:454-6.
44. Meerschaert J, Furie MB. The adhesion molecules used by monocytes for migration across endothelium include CD11a/CD18, CD11b/CD18, and VLA-4 on monocytes and ICAM-1, VCAM-1, and other ligands on endothelium. *J Immunol* 1995;154:4099-112.
45. van Halm VP, van Denderen JC, Peters MJ, Twisk JW, van der Paardt M, van der Horst-Bruinsma IE, et al. Increased disease activity is associated with a deteriorated lipid profile in patients with ankylosing spondylitis. *Ann Rheum Dis* 2006;65:1473-7.
46. Papadakis JA, Sidiropoulos PI, Karvounaris SA, Vrentzos GE, Spanakis EK, Ganotakis ES, et al. High prevalence of metabolic syndrome and cardiovascular risk factors in men with ankylosing spondylitis on anti-TNF $\alpha$  treatment: correlation with disease activity. *Clin Exp Rheumatol* 2009;27:292-8.
47. Hwang SJ, Ballantyne CM, Sharrett AR, Smith LC, Davis CE, Gotto AM Jr, et al. Circulating adhesion molecules VCAM-1, ICAM-1, and E-selectin in carotid atherosclerosis and incident coronary heart disease cases: the Atherosclerosis Risk In Communities (ARIC) study. *Circulation* 1997;96:4219-25.
48. Garlanda C, Bottazzi B, Moalli F, Deban L, Molla F, Latini R, et al. Pentraxins and atherosclerosis: the role of PTX3. *Curr Pharm Des* 2011;17:38-46.
49. Jenny N, Arnold AM, Kuller LH, Tracy RP, Psaty BM. Association of pentraxin 3 with cardiovascular disease and all-cause death. The Cardiovascular Health Study. *Arterioscler Thromb Vasc Biol* 2009;29:594-9.
50. Rothe G, Gabriel H, Kovacs E, Klucken J, Stöhr J, Kindermann W, et al. Peripheral blood mononuclear phagocyte subpopulations as cellular markers in hypercholesterolemia. *Arterioscler Thromb Vasc Biol* 1996;16:1437-47.