

Toll-like receptor 3 and interleukin 1 β expression in CD34⁺ cells from patients with rheumatoid arthritis: association with inflammation and vascular involvement

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Abstract

Objective

Circulating proangiogenic haematopoietic cells (PHCs), including CD34⁺ cells, play an important role in endothelial homeostasis. Among PHCs, CD34⁺ cells are the largest cell population, thus, much of the regenerative/repairative potential of PHCs may be attributed to CD34⁺ cells. Our aim was to determine the association between inflammation and CD34⁺ cell number, intracellular levels of reactive oxygen species (ROS) and expression of Toll-like receptor 3 (TLR3) and interleukin 1 β (IL-1 β), arterial stiffness (AS) indices, and carotid intima-media thickness (cIMT) in patients affected by rheumatoid arthritis (RA).

Methods

CD34⁺ cells were isolated from 24 RA patients and 26 matched controls. ROS levels, TLR3 and IL-1 β expression were measured. C-reactive protein (CRP), fibrinogen, AS, and cIMT were also evaluated.

Results

CD34⁺ count was lower in RA patients as compared to controls. In CD34⁺ cells from RA patients, ROS, TLR3 and IL-1 β expressions were increased compared to controls. In RA patients, we found higher CRP and fibrinogen levels, and higher values of Pulse Wave Velocity (PWV) and Augmentation Index (AIx), both AS indices, and of cIMT. CD34⁺ cell numbers were inversely correlated with CRP, TLR3, IL-1 β , ROS, and AS indices. TLR3 levels were related to CRP, IL-1 β , fibrinogen and ROS. IL-1 β levels were correlated with expression of CRP, ROS, and PWV.

Conclusion

Inflammatory status in RA is associated with an increased expression of TLR3 and of IL-1 β in CD34⁺ cells, which appear to affect cell number. These new findings suggest a perspective on accelerated atherosclerosis and vascular damage in RA.

Key words

Toll-like receptor 3, interleukin 1 β , circulating progenitor cells, preclinical atherosclerosis, arterial stiffness, rheumatoid arthritis

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Introduction

Circulating proangiogenic haematopoietic cells (PHCs) (1, 2) are multipotent circulating cells derived from bone marrow that have been proven capable of angiogenic and reparative properties *in vivo*. PHCs are a heterogeneous population of cells in different states of maturation with the ability to differentiate into cell types of different organs and systems, including cardiomyocytes, smooth muscle cells, and endothelial progenitor cells (3, 4). They are different from endothelial forming colony cells and, although to date it is not clear if they can give rise to mature endothelial cells (ECs) *in vivo*, it is currently accepted that PHCs can work as proangiogenic support cells, maintaining importance in terms of regenerative/reparative potential and prognostic value (4). PHCs have been shown to contribute to postnatal vasculogenesis and vascular damage repair both directly and via paracrine effects (1), participating in healthy and damaged endothelial turnover and angiogenesis. They are negatively affected by risk factors for cardiovascular disease (CVD) and positively by changes in lifestyle (5); consequently, their number is considered an independent predictor of CVD, and morbidity/mortality also for non-CV causes, even among healthy subjects and in the elderly (6-8). Moreover, CD34⁺ cells are the largest cell population (including also their subsets, multiple positive phenotypes CD34⁺/CD133⁺, CD34⁺/KDR⁺, and CD34⁺/CD133⁺/KDR⁺) among PHCs; much of the regenerative/reparative potential of PHCs may be attributed to CD34⁺ cells.

Rheumatoid arthritis (RA) represents a human model of chronic inflammatory disease presenting with accelerated atherosclerosis and increased rate of cardiovascular disease (9-10). The molecular mechanisms underlying the increased incidence or accelerated onset of CVD in patients with RA have not been fully clarified; systemic inflammation may have a key role, since it was shown that the pathogenesis and aetiology of RA and CVD share many common cellular and molecular mediators (11). Several pro-inflammatory cytokines, including TNF- α , C-reactive

Protein (CRP) and interleukin-6 released from synovia into the systemic circulation, affect endothelium homeostasis promoting its pro-atherogenic activation and dysfunction (12).

It has already been shown in RA that PHCs are recruited by specific adhesion molecules into the joints, participating in synovial neoangiogenesis (13). While PHCs are recruited into the rheumatic tissue, depletion may occur in peripheral blood, potentially compromising endothelial repair and leading to vascular lesions (14, 15). Although it has been hypothesised that in this condition cell decrease may contribute to the increased cardiovascular risk in RA, additional factors appear capable of modulating the number and activity of progenitor cells in chronic inflammatory disorders (16, 17).

Toll-like receptor-3 (TLR3) is a pattern recognition receptor that plays an important role in the activation of innate immunity and priming of adaptive immunity against pathogens and "danger signals", including factors released from stressed or damaged cells and tissue (18, 19). TLR3 and interleukin 1- β (IL1- β) have been detected in EPCs from human umbilical cord blood, and their involvement in cell cycle progression and proliferation has been suggested (20). IL1- β , which is synthesised as a precursor peptide, is activated in response to various pro-inflammatory stimuli, also via TLRs (21-23). In particular, it has been shown that in cultured EPCs from human umbilical cord blood, the activation of TLR3 by the synthetic double stranded RNA analogue polyriboinosinic-ribocytidylic acid (polyI:C) inhibits cell cycle progression and induces caspase-independent cell apoptosis, which is, at least in part, related to increased expression of IL1- β (20); additionally, TLR3 activation by polyI:C in cultured PHCs was found to increase reactive oxygen species (ROS) production and cell apoptosis (24). The activation of TLR3 is consequently thought to affect progenitor cell number and function and may consequently compromise vascular repair and angiogenesis (25).

In the present study, we analysed expression of TLR3 and IL1- β in CD34⁺

Competing interests: none declared.

cells from untreated patients affected by RA, and without additional risk factors for atherosclerosis or CVD. An observational cross sectional study was designed to investigate the relationships between CD34⁺ cell number, intracellular ROS levels, TLR3 and IL1- β expression, and systemic inflammation. Arterial stiffness indices (AS) and carotid intima-media thickness (cIMT), both patterns of preclinical atherosclerosis (26), were also evaluated in relation to inflammatory markers, CD34⁺ cell number, ROS, TLR3 and IL1- β expression.

Materials and methods

Subjects

Between October 2012 and May 2013, 597 outpatients were examined for the first time at the Rheumatology Division of the University of Messina and were referred for a clinical and instrumental screening; according to inclusion/exclusion criteria, only 24 subjects (15 men and 9 women) were considered eligible for this study: to be recruited for the study, subjects needed to be newly diagnosed, untreated, not have additional risk factors for atherosclerosis or CVD, and meet the retrospective application of the 1987 revised RA criteria of the American Rheumatism Association (27); additionally, they should never have been treated with immunosuppressive drugs, long-term corticosteroids and/or NSAIDs nor DMARDs. Subjects with co-morbidities, such as diabetes mellitus, dyslipidaemia (defined as plasma levels of cholesterol ≥ 230 mg/dl or low-density lipoprotein cholesterol [LDL-C] ≥ 160 mg/dl, or triglycerides ≥ 250 mg/dl), hypertension (defined as systolic blood pressure [SBP] ≥ 140 mmHg and/or diastolic blood pressure [DBP] ≥ 90 mmHg) were excluded from the study. Smokers were also excluded from the study. Women taking hormone-based therapy were not included in the study. Thyroid, liver or kidney diseases, body mass index (BMI) ≥ 30 , alcohol consumption, abnormal electrocardiographic or echocardiographic (left ventricular ejection function, left ventricular regional function) pattern, and clinical history of CVD were also considered as exclusion criteria. No

subjects were taking any drugs, including antioxidant vitamins. Patients previously treated with NSAIDs should not take drugs for at least two weeks before inclusion.

Twenty-six subjects (15 men and 11 women) matched for age and gender were enrolled from hospital personnel as control subjects. After inclusion in the study, patients and controls underwent blood sampling and instrumental examination as described below; patients were then referred to the rheumatology clinic for clinical and therapeutic follow-up.

Written informed consent was obtained from all subjects according to the Helsinki declaration and the study was approved by the Ethics Committee of the University of Messina.

Methods

All chemical analyses were performed at the medical centre after overnight fasting. Plasma lipids, glucose, fibrinogen, and rheumatoid factor (RF) were determined by routine methods. CRP and anti-cyclic citrullinated peptide antibodies (aCCP) were determined by a commercially available ELISA kit.

Fresh blood flow cytometry (FACS Calibur; Becton Dickinson and Co., Franklin Lakes, NJ, USA) was used to identify the cells. Circulating cells that expressed the stem cell antigen CD34 were defined as progenitor cells, and estimated and counted (cells/ μ L).

Expression of TLR3 and IL-1 β -mRNA in circulating CD34⁺ cells was evaluated after the enrichment of the samples in CD34⁺ cells by immunomagnetic sorting with the MiniMACS system (Miltenyi Biotec Inc, Auburn, CA, USA).

To perform molecular analyses on CD34⁺ cells, 50 ml of blood was collected from each subject and immediately processed; mononuclear cells were separated from other blood components using a gradient separation method. CD34⁺ cell sample enrichment, mRNA extraction and reverse transcription, polymerase chain reaction, and validation procedure have already been explained in detail elsewhere (28); briefly, after the isolation of mononuclear cells by density centrifugation and after enrichment of the samples in CD34⁺ cells

by the MiniMACS system (Miltenyi Biotec Inc., CA, USA), expression of TLR3 and IL1- β was evaluated. Cell enrichment was validated by flow cytometry, confirming that at least 90% of separated cells were CD34⁺ (Fig. 1). Total RNA was then extracted, quantified and retro-transcribed. cDNA was used to measure gene expression of TLR3 and IL1- β by real time polymerase chain reaction (RT-PCR), using β -actin as endogenous controls for final normalisation; relative expression was measured using the $2^{-\Delta\Delta C_t}$ method. For this method, average values of TLR3 and IL1- β in samples from all control subjects were considered as the calibrator (1 $\times$ sample). Results were expressed as an n-fold difference relative to the mean value (relative expression levels). ROS generation in CD34⁺ cell-enriched samples was assessed using 2,7-dichlorofluorescein diacetate (DCFH-DA), by fluorimetric method (29). Data are expressed as fluorescence intensity relative units (FU).

Measurement of cIMT and AS indices

Carotid echo Doppler scan and arterial stiffness assessment were performed using Aloka ProSound ALPHA 10 with a 7–15 MHz linear array transducer. Semi-automated cIMT was evaluated bilaterally on the far wall in the areas of the common carotid (1cm proximal to the carotid bulb), the carotid bifurcation (1cm proximal to the flow divider) and the internal carotid artery (1cm distal to the flow divider). According to ESC/ESH guidelines, we considered as carotid wall thickening a cIMT ≥ 0.9 mm or plaque. Augmentation Index (AIx) and Pulse wave velocity (PWV) as AS indices were measured automatically by “eTRACKING” software. An extensive explanation of the mechanical and physical bases of this analysis has previously been provided (30).

Statistical analysis

The Kolmogorov Smirnov test verified that some variables had a non-normal distribution; consequently, given also the small size of our sample, we chose to use a permutation test-based analysis. This subset of non parametric statistics, widely used in biomedical

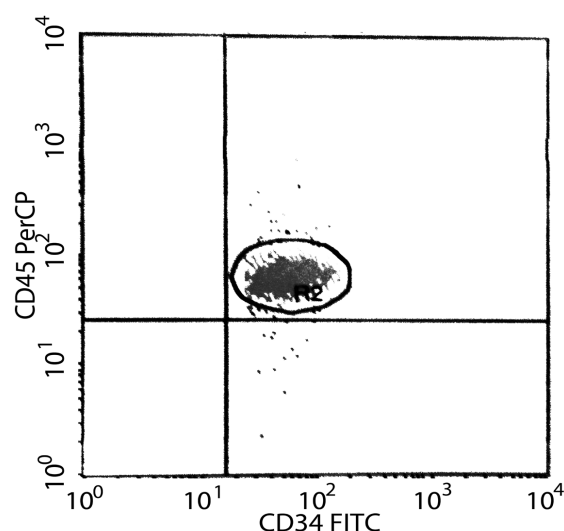


Fig. 1. Purity plot of CD34⁺ cells enriched sample

Table I. Characteristics of the study population.

Number Gender (m/f)	RA 24 15/9		Controls 26 15/11		<i>p</i> -value NS NS	
	Mean $\pm$ SD	Median (IQR)	Mean $\pm$ SD	Median (IQR)	NPCT	Mann-Whitney
Age (years)	48.5 $\pm$ 7.14	48 (11)	46.4 $\pm$ 2.4	46.5 (1.75)	NS	NS
BMI (kg/m ²)	25.1 $\pm$ 2.6	25 (4.3)	24.3 $\pm$ 2.1	24.5 (3.4)	NS	NS
SBP (mmHg)	125.8 $\pm$ 6.6	126 (10)	122.7 $\pm$ 9.5	122.5 (10)	NS	NS
DBP (mmHg)	73.8 $\pm$ 4.4	70 (10)	72.5 $\pm$ 6.3	75 (10)	NS	NS
TC (mg/dl)	196.5 $\pm$ 38.5	190 (53)	181.3 $\pm$ 63.9	180 (40)	NS	NS
HDL-C (mg/dl)	52.3 $\pm$ 11.1	52 (13.5)	51.5 $\pm$ 9.2	50 (12)	NS	NS
TG (mg/dl)	123.3 $\pm$ 48	125 (65)	110.7 $\pm$ 22.2	111.5 (32.2)	NS	NS
LDL-C (mg/dl)	126.6 $\pm$ 30.8	122 (39)	112.1 $\pm$ 24	118.2 (46)	NS	NS
Glucose (mg/dl)	88.5 $\pm$ 10.9	88 (18.5)	87.6 $\pm$ 7.1	88 (11.2)	NS	NS
Hs-CRP (mg/dl)	5.33 $\pm$ 4.31	4.55 (4.25)	0.62 $\pm$ 0.3	0.62 (0.8)	0.001	0.001
Fibrinogen (mg/dl)	389.2 $\pm$ 52	398 (62.5)	260 $\pm$ 57.7	256 (109)	0.001	0.001
CD34 ⁺ (cells/ μ L)	1.90 $\pm$ 0.63	1.85 (0.95)	2.52 $\pm$ 1.14	2.6 (2)	0.01	0.03
TLR3 (n-fold)	1.54 $\pm$ 0.48	1.45 (0.48)	1 $\pm$ 0.3	1 (0.4)	0.001	0.001
IL-1 β (n-fold)	1.65 $\pm$ 0.37	1.76 (0.34)	1 $\pm$ 0.25	1 (0.5)	0.001	0.001
ROS (FU)	114.2 $\pm$ 62.3	86.4 (108.9)	47.7 $\pm$ 11.9	51 (10.4)	0.001	0.001
AIx (%)	10.21 $\pm$ 7.31	8.8 (13.4)	-3.57 $\pm$ 4.5	-3.0 (5.26)	0.001	0.001
PWV (m/s)	7.48 $\pm$ 1.65	7.3 (2.2)	4.54 $\pm$ 0.8	4.85 (1.1)	0.001	0.001
IMT (mm)	1.11 $\pm$ 0.14	1.15 (0.25)	0.77 $\pm$ 0.16	0.79 (0.24)	0.001	0.001

Values are mean $\pm$ SD. BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; TC: total cholesterol; HDL-C: high density lipoprotein-cholesterol; TG: triglycerides; LDL-C: low density lipoprotein cholesterol; Hs-CRP: high sensitivity C-reactive protein.; AIx: augmentation index; PWV: pulse wave velocity; cIMT: carotid intima-media thickness; Toll-like receptor 3; IL-1 β : Interleukin 1 β ; *p*-value: *p*-value level for NPC-test/Mann-Whitney U-test, RA patients vs. controls.

research, is considered preferable to the classic non-parametric approach (31), since it is based on more realistic foundations, it is intrinsically robust, the resulting inferences are credible and it estimates the whole data distribution exploiting all information contained in the sample (31). Accordingly, data were expressed as mean $\pm$ standard deviation (SD). Comparisons were carried out by the Non

Parametric Combination test (NPC test), which is based on a simulation or resampling procedure, conditional on the data, providing a simulated estimate of the permutation distribution of any statistic (32).

Moreover, in order to verify the results obtained with this approach, we integrated the statistical analysis with a traditional non-parametric approach (median and IQR), and, consequently,

the comparisons between variables were carried out by the Mann-Whitney U-test. Correlations among variables were assessed by Spearman's test. To assess the contribution of each variable on study variables a linear, step-wise, multivariate regression analysis was performed that allows considering continuous and categorical variables together on the whole study population. A two-tailed alpha of 0.05 was used to denote statistical significance. SPSS statistical package, ver. 17.0 (Chicago, IL, USA), was used to perform statistical analyses, along with the NPC test 2.0 – Statistical software for multivariate permutation tests (Methodologica srl, Treviso, Italy).

Results

Table I shows the baseline characteristics of the study groups. No difference was detected with regard to age, BMI, gender, BP, glucose, lipids. Fibrinogen, CRP and ESR were significantly higher in RA patients compared to controls ($p < 0.001$). Antibody status was also evaluated: 18 out of 24 patients were RF positive; of these, 11 were also aCCP positive; 6 patients were both RF and aCCP negative.

PWV and AIx were significantly higher in RA patients compared to controls (both $p < 0.001$), also cIMT values were increased compared to controls ($p < 0.001$) and, on average, above 0.9 mm, considered the upper reference limit for preclinical atherosclerosis according to ESH-ESC guidelines (33).

Figure 2 shows box and whiskers plots for PWV, AIx, TLR3, IL-1 β , ROS and CD34⁺ cells in RA and control subjects. In RA patients, the number of CD34⁺ cells was significantly lower compared to controls ($p < 0.001$), and cells isolated from RA patients displayed higher levels of ROS ($p < 0.001$). In RA patients, expression of TLR3 and IL-1 β mRNA was higher than in controls (both $p < 0.001$).

In RA patients, a positive correlation was found between TLR3 and: CRP ($rs = 0.435$ $p < 0.05$), IL-1 β ($rs = 0.687$, $p < 0.001$), fibrinogen ($rs = 0.437$, $p < 0.05$), and ROS ($rs = 0.501$ $p < 0.02$). IL-1 β levels were also correlated with expression of: ROS ($rs = 0.482$ $p < 0.02$),

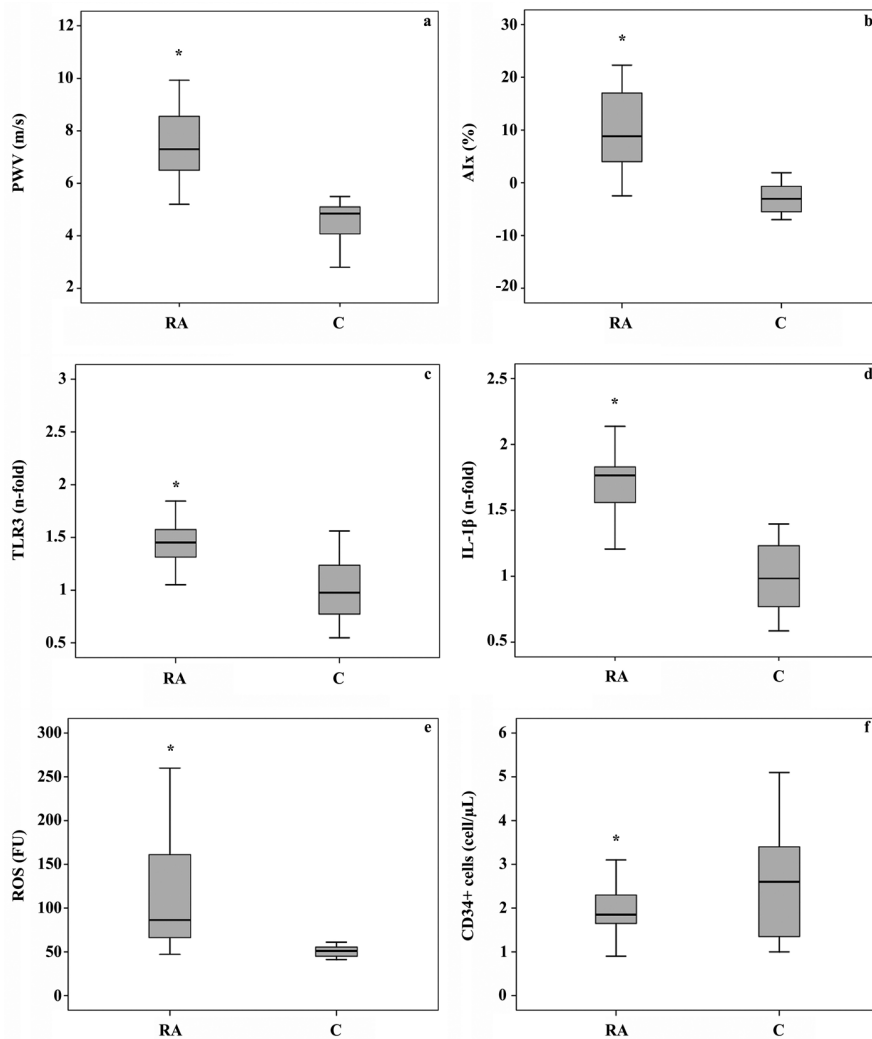


Fig. 2. Box and whiskers plots for PWV (a), AIx (b), TLR3 (c), IL-1 β (d), ROS (e) and CD34⁺ (f) in RA patients and controls. * $p < 0.001$ vs. controls. Solid horizontal lines: median values; error bars: 95% confidence intervals; shaded area: interquartile range.

CRP ($rs = 0.530$, $p < 0.02$), and PWV ($rs = 0.434$, $p < 0.05$). In RA, no correlation was found between vascular parameters (AS indices and cIMT) and CRP. We verified whether any correlation exists between CD34⁺ cell number, inflammatory markers (CRP, Fibrinogen), TLR3 and IL-1 β levels, ROS, AS indices (PWV and AIx) in RA patients. In RA patients, the number of CD34⁺ cells correlated inversely with CRP ($rs = -0.524$, $p < 0.01$), Fibrinogen ($rs = -0.414$, $p < 0.05$), IL-1 β ($rs = -0.673$, $p < 0.001$), TLR3 ($rs = -0.629$, $p < 0.001$), ROS ($rs = -0.454$, $p < 0.01$), and AIx ($rs = -0.433$, $p < 0.05$). Dependence analysis suggested that the main variable associated to CD34⁺ cell number was IL-1 β ($\beta = -0.772$, $p < 0.001$), which in turn appeared associated to TLR3 ($\beta = 0.786$,

$p < 0.001$) and CRP ($\beta = 0.222$, $p < 0.01$); CRP also appeared to affect TLR3 ($\beta = 0.509$, $p < 0.001$). Correlations between CD34⁺ cell number and CRP, ROS, TLR3 and IL-1 β are shown in Figure 3.

Discussion

The mechanisms behind the higher incidence of coronary heart disease in RA patients are to date not fully understood, but it is likely that incidence may be linked to vascular inflammation and vascular endothelial injury, and consequently to accelerated atherosclerosis, which are common in RA patients. Homeostasis of the intimal layer relies on the contribution of circulating cells that participate in the turnover of healthy and damaged endothelium, as well as

in angiogenesis (34). However, in the last few years, several cell phenotypes have been proposed as EPCs and considered as potential spare cells, able to participate in the turnover of healthy and damaged vascular endothelium; their number has been considered as an independent predictor of CV risk, even in healthy subjects (3, 4, 6, 7, 35). The question of which cell phenotype better identifies the “true” circulating EPC remains unsolved, since the more widely studied PHC phenotypes do not give rise to mature ECs and are different from endothelial forming colony cells (2, 36). However, it is currently accepted that, although PHCs are derived from hematopoietic lineage, they can work as proangiogenic support cells, maintaining importance as regenerative/reparative potential, and prognostic value (2, 4, 28, 37, 38). We have chosen to limit the investigation to CD34⁺ cell phenotype, since, apart from this subset, the other main PHC phenotypes are relatively rare in peripheral blood. Thus, to extend molecular studies to each PHC phenotype would require considerable effort and large amounts of blood from each patient to isolate a reasonable number of cells and allow enzyme expression assessment; moreover, it has recently been suggested that expression of CD34 surface antigen may display an important role in angiogenic cell properties (39).

Among the factors that could lead to a reduction of cells, inflammation may play a prominent role. It has been well established that in an inflammatory status EPC function is impaired, and several inflammatory mediators play a dual role in EPC mobilisation (40): low-grade inflammation induces EPC mobilisation, whereas high-grade and prolonged inflammatory stimulation has the opposite effect. The effects of long-term proinflammatory stimulation on bone marrow are not well known, but it is likely that it may lead to exhaustion of the PHC pool with release of immature or dysfunctional progenitors. In accordance with previous studies, we found a reduced CD34⁺ cell number in patients affected by RA, which appears directly related to the increased levels of CRP. Furthermore,

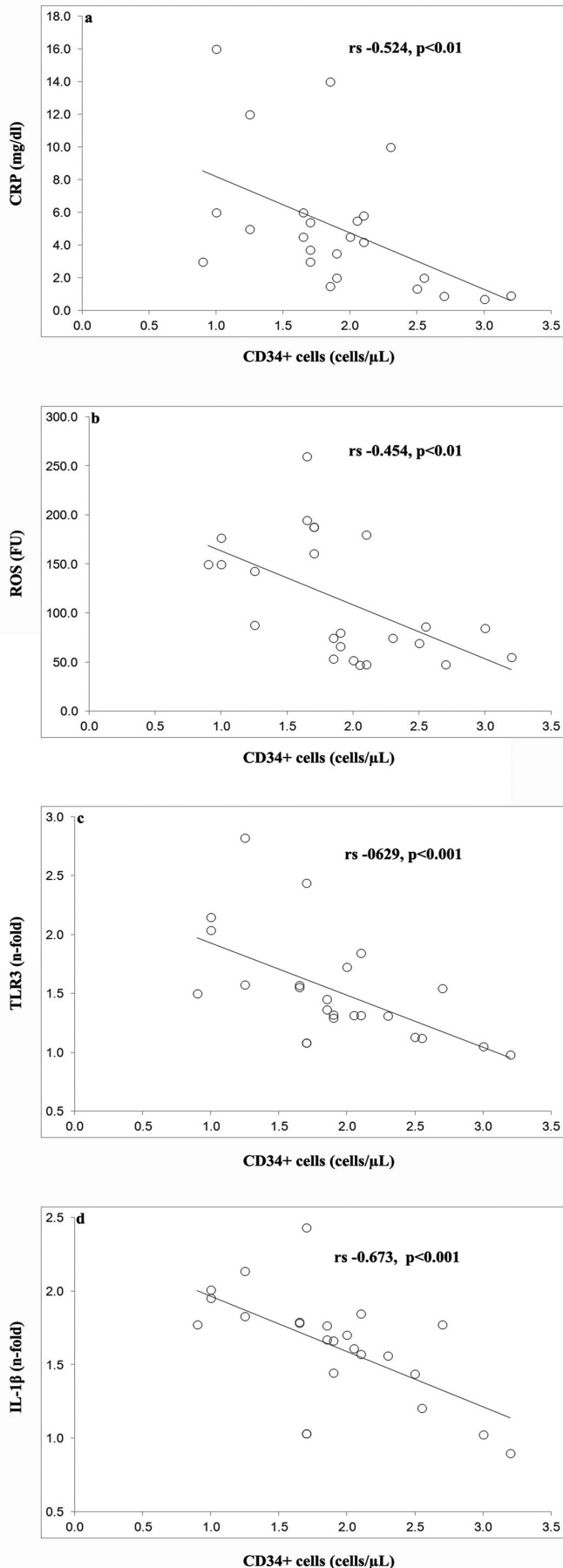


Fig. 3. Spearman's correlation between CD34 $^{+}$ cell number and CRP (a), ROS (b), TLR3 (c), and IL-1 β (d) in RA subjects; Spearman's coefficient (rs) is shown with its significance (p).

multiple regression analysis confirmed a significant role of CRP in impairing CD34 $^{+}$ cell number.

CRP, an acute phase protein predominantly produced in the liver, is to date considered a potential causal factor in atherogenesis and is independently associated with increased risk of fatal and non fatal cardiovascular events, even in apparently healthy individuals (41). CRP is capable of inhibiting EPC differentiation, survival and function *in vitro* (42); it impairs EPC antioxidant potential, and may promote EPC sensitivity toward oxidant-mediated apoptosis and telomerase inactivation (43). However, CRP, in addition to having a direct effect on cells, could also alter the number, increasing expression of TLR3 and IL-1 β . TLRs are expressed on several immune cells, including macrophages, dendritic cells, B cells, specific types of T cells, and even on non-immune cells such as fibroblasts, and epithelial cells (44). These receptors recognise an enormously diverse range of ligands, including exogenous molecules derived from invading microbes, pathogen-associated molecular patterns (PAMPs), and endogenous molecules created or up-regulated upon tissue injury, damage-associated molecular patterns (DAMPs). Ligand binding induces receptor conformational changes and results in the recruitment and/or activation of adaptor molecules that initiate a cascade of inflammatory signalling events (19). Yang *et al.* showed that TLR3 and IL-1 β are functionally expressed in EPCs, and activation impairs cell proliferation, inhibits cell cycle progression and induces apoptosis (20).

The increased expression of TLR3 in inflammatory conditions, including RA, has been already reported in several cell types such as dendritic cells, synovial fibroblasts, macrophages, and endothelial cells (45, 46); furthermore, it has been reported that inflammatory molecules such as IFN- α are capable of up-regulating TLR3 transcription in mesenchymal stromal cells (46).

In this study, we found an increased expression of TLR3 and IL-1 β also in CD34 $^{+}$ cells from RA patients, and that increase was related to higher CRP plasma levels. These observations, there-

fore, could confirm a modulation of inflammation on the expression of TLR3 also in circulating CD34⁺ cells.

The activation of TLR3 generally leads to an inflammatory cascade and release of cytokines including IL-1 β (19). IL-1 β , a pleiotropic pro-inflammatory cytokine, is involved in apoptosis of different cell types (47, 48) and could inhibit cell proliferation in endothelial progenitors (20); moreover, it has recently been shown that TLR3 activation in cord blood-derived endothelial cell causes cell proliferation inhibition, cell cycle entry modifications, impairment of *in vitro* angiogenic properties and pro-inflammatory cytokines production (25). Consistent with previous observations, we found that decreased levels of circulating CD34⁺ cells were related to a higher expression of IL-1 β . Thus, we could speculate that TLR3 activation leads to *de novo* cytokine generation and may strengthen a pre-existing inflammatory status associated with rheumatic synovium (45). Therefore, TLR3 could indirectly alter the function of PHCs (19), reducing their number.

Additionally, TLR3 could affect the cells through the stimulation of ROS production. RA patients displayed higher ROS levels which were related to expression of TLR3 and IL-1 β and were also related to decreased number of cells. Zimmer *et al.* reported that ROS production was increased by TLR3 stimulation and remarked that the rapid formation of ROS in response to the activation of TLR3 by immune cells is an important, defensive mechanism against pathogens (24). Although PHCs have an excellent anti-oxidant system, it has been reported that its function can be impaired by inflammation (17).

Our results, although they cannot provide a mechanistic model by which the inflammatory status could lead to the impairment of circulating CD34⁺ cells, appear to suggest that CRP elevation may be the trigger that promotes overexpression of TLR3 and, consequently, of IL-1 β , which in turn was found to be associated to cell decrease. We also confirmed that RA patients present with altered AS indices. The association between inflammation and arterial stiffening has already been suggested by

several studies in patients with chronic inflammatory diseases, including RA (17, 26, 49); AS is widely established as an integrated marker of arterial disease, and a valuable predictor for future cardiovascular disease and death, and is considered the first reversible step in atherogenesis (50). It is likely that a clinical condition characterised by a chronic inflammatory status and impaired reparative/regenerative endothelial potential also due to reduced PHC cell number may at least in part explain the accelerated progression of endothelial functional damage (arterial stiffening) to more advanced vascular damage (intima-media thickening) and development of CVD also in patients without personal risk factors for CVD.

This study, however, presents several limitations. The first limitation is the small sample size; according to inclusion/exclusion criteria we enrolled only 24 patients. Second, we investigated the association between inflammation and TLR3 and IL-1 β levels in CD34⁺ cells without a focused investigation on mechanistic and pathophysiological aspects that could explain this association, but this was not the purpose of the study.

Conclusion

In conclusion, these data suggest that chronic inflammatory status is associated to overexpression of TLR3 and IL-1 β in CD34⁺ cells from RA patients. Furthermore, the increased CD34⁺ cell expression of IL-1 β and TLR3 appears to be linked to reduced circulating cell number, and consequently to an impaired reparative/regenerative endothelial potential. It is likely that this novel association may at least in part contribute to explaining the increase of cardiovascular morbidity and mortality in patients suffering from RA. Further studies on larger sample sizes could clarify whether the modulation of TLR3 or IL-1 β expression in PHCs, also by therapy or specific drugs, may modify CV risk in patients affected by RA.

References

1. YAMAGUCHI Y, KUWANA M: Proangiogenic hematopoietic cells of monocytic origin: roles in vascular regeneration and pathogenic processes of systemic sclerosis. *Histol Histopathol* 2013; 28: 175-83.

2. RICHARDSON MR, YODER MC: Endothelial progenitor cells: quo vadis? *J Mol Cell Cardiol* 2011; 50: 266-72.
3. STEINMETZ M, NICKENIG G, WERNER N: Endothelial-regenerating cells: an expanding universe. *Hypertension* 2010; 55: 593-9.
4. DE BOER HC, HOVENS MM, VAN OEVEREN-RIETDIJK AM *et al.*: Human CD34⁺/KDR⁺ cells are generated from circulating CD34⁺ cells after immobilization on activated platelets. *Arterioscler Thromb Vasc Biol* 2011; 31: 408-15.
5. DI STEFANO R, FELICE F, FERIANI R, BALBARINI A: Endothelial progenitor cells, cardiovascular risk factors and lifestyle modifications. *Intern Emerg Med* 2013; 8 (Suppl. 1): S47-9.
6. WERNER N, KOSIOL S, SCHIEGL T *et al.*: Circulating endothelial progenitor cells and cardiovascular outcomes. *N Engl J Med* 2005; 353: 999-1007.
7. HILL JM, ZALOS G, HALCOX JP *et al.*: Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N Engl J Med* 2003; 348: 593-600.
8. MANDRAFFINO G, SARDO MA, RIGGIO S *et al.*: Circulating progenitor cells and the elderly: a seven-year observational study. *Exp Gerontol* 2012; 47: 394-400.
9. WARRINGTON KJ, KENT PD, FRYE RL *et al.*: Rheumatoid arthritis is an independent risk factor for multi-vessel coronary artery disease: a case control study. *Arthritis Res Ther* 2005; 7: R984-91.
10. MEUNE C, TOUZE E, TRINQUART L, ALLANORE Y: Trends in cardiovascular mortality in patients with rheumatoid arthritis over 50 years: a systematic review and meta-analysis of cohort studies. *Rheumatology (Oxford)* 2009; 48: 1309-13.
11. KU IA, IMBODEN JB, HSUE PY, GANZ P: Rheumatoid arthritis: model of systemic inflammation driving atherosclerosis. *Circ J* 2009; 73: 977-85.
12. SATTAR N, MCCAREY DW, CAPELL H, MCINNES IB: Explaining how "high-grade" systemic inflammation accelerates vascular risk in rheumatoid arthritis. *Circulation* 2003; 108: 2957-63.
13. SILVERMAN MD, HAAS CS, RAD AM, ARBAB AS, KOCH AE: The role of vascular cell adhesion molecule 1/ very late activation antigen 4 in endothelial progenitor cell recruitment to rheumatoid arthritis synovium. *Arthritis Rheum* 2007; 56: 1817-26.
14. HERBRIG K, HAENSEL S, OELSCHLAEGEL U, PISTROSCH F, FOERSTER S, PASSAUER J: Endothelial dysfunction in patients with rheumatoid arthritis is associated with a reduced number and impaired function of endothelial progenitor cells. *Ann Rheum Dis* 2006; 65: 157-63.
15. GRISAR J, ALETAHA D, STEINER CW *et al.*: Depletion of endothelial progenitor cells in the peripheral blood of patients with rheumatoid arthritis. *Circulation* 2005; 111: 204-11.
16. WESTERWEEL PE, VERHAAR MC: Endothelial progenitor cell dysfunction in rheumatic disease. *Nat Rev Rheumatol* 2009; 5: 332-40.
17. LO GULLO A, MANDRAFFINO G, SARDO M *et al.*: Circulating progenitor cells in rheu-

- matoid arthritis: association with inflammation and oxidative stress. *Scand J Rheumatol* 2014; 43: 184-93.
18. ALEXOPOULOU L, HOLT AC, MEDZHITOV R, FLAVELL RA: Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* 2001; 413: 732-8.
 19. MONACO C, TERRANDO N, MIDWOOD KS: Toll-like receptor signaling: common pathways that drive cardiovascular disease and rheumatoid arthritis. *Arthritis Care Res (Hoboken)* 2011; 63: 500-11.
 20. YANG M, XIAO Z, LV Q *et al.*: The functional expression of TLR3 in EPCs impairs cell proliferation by induction of cell apoptosis and cell cycle progress inhibition. *Int Immunopharmacol* 2011; 11: 2118-24.
 21. MARTINON F, TSCHOPP J: Inflammatory caspases and inflammasomes: master switches of inflammation. *Cell Death Differ* 2007; 14: 10-22.
 22. GABAY C, LAMACCHIA C, PALMER G: IL-1 pathways in inflammation and human diseases. *Nat Rev Rheumatol* 2010; 6: 232-41.
 23. MAELFAIT J, VERCAMMEN E, JANSSENS S *et al.*: Stimulation of Toll-like receptor 3 and 4 induces interleukin-1beta maturation by caspase-8. *J Exp Med* 2008 Sep; 205: 1967-73.
 24. ZIMMER S, STEINMETZ M, ASDONK T *et al.*: Activation of endothelial toll-like receptor 3 impairs endothelial function. *Circ Res* 2011; 108: 1358-66.
 25. GRELLIER A, CRAS A, BALITRAND N *et al.*: Toll-like receptor 3 regulates cord blood-derived endothelial cell function in vitro and in vivo. *Angiogenesis* 2013; 16: 821-36.
 26. PROVAN SA, ANGEL K, SEMBAG *et al.*: Early prediction of increased arterial stiffness in patients with chronic inflammation: a 15-year followup study of 108 patients with rheumatoid arthritis. *J Rheumatol* 2011; 38: 606-12.
 27. ARNETT FC, EDWORTHY SM, BLOCH DA *et al.*: The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988; 31: 315-24.
 28. MANDRAFFINO G, SARDO MA, RIGGIO S *et al.*: Circulating progenitor cells are increased in newly diagnosed untreated hypertensive patients with arterial stiffening but normal carotid intima-media thickness. *Hypertens Res* 2011; 34: 876-83.
 29. LEBEL CP, ISCHIROPOULOS H, BONDY SC: Evaluation of the probe 2',7'-dichlorofluorescein as an indicator of reactive oxygen species formation and oxidative stress. *Chem Res Toxicol* 1992; 5: 227-31.
 30. RIGGIO S, MANDRAFFINO G, SARDO MA *et al.*: Pulse wave velocity and augmentation index, but not intima-media thickness, are early indicators of vascular damage in hypercholesterolemic children. *Eur J Clin Invest* 2010; 40: 250-7.
 31. PESARIN F, SALMASO L: Permutation Tests for Complex Data: Theory, Applications and Software, Chichester, Wiley, 2010.
 32. PESARIN F (Ed.): Multivariate Permutation Tests with Applications in Biostatistics, Chichester, Wiley, 2001.
 33. MANCIA G, DE BACKER G, DOMINICZAK A *et al.*: 2007 Guidelines for the Management of Arterial Hypertension: The Task Force for the Management of Arterial Hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). *J Hypertens* 2007; 25: 1105-87.
 34. FADINI GP, DE KREUTZENBERG S, AGOSTINI C *et al.*: Low CD34⁺ cell count and metabolic syndrome synergistically increase the risk of adverse outcomes. *Atherosclerosis* 2009; 207: 213-9.
 35. PESCE M, BURBA I, GAMBINI E, PRANDI F, POMPILIO G, CAPOGROSSI MC: Endothelial and cardiac progenitors: boosting, conditioning and (re)programming for cardiovascular repair. *Pharmacol Ther* 2011; 129: 50-61.
 36. HIRSCHI KK, INGRAM DA, YODER MC: Assessing identity, phenotype, and fate of endothelial progenitor cells. *Arterioscler Thromb Vasc Biol* 2008; 28: 1584-95.
 37. REYES M, DUDEK A, JAHAGIRDAR B, KOODIE L, MARKER PH, VERFAILLIE CM: Origin of endothelial progenitors in human postnatal bone marrow. *J Clin Invest* 2002; 109: 337-46.
 38. FADINI GP, MARUYAMA S, OZAKI T *et al.*: Circulating progenitor cell count for cardiovascular risk stratification: a pooled analysis. *PLoS One* 2010; 5: e11488.
 39. SIEMERINK MJ, KLAASSEN I, VOGELS IM, GRIFFIOEN AW, VAN NOORDEN CJ, SCHLINGEMANN RO: CD34 marks angiogenic tip cells in human vascular endothelial cell cultures. *Angiogenesis* 2012; 15: 151-63.
 40. TOUSOULIS D, ANDREOU I, ANTONIADES C, TENTOLOURIS C, STEFANADIS C: Role of inflammation and oxidative stress in endothelial progenitor cell function and mobilization: therapeutic implications for cardiovascular diseases. *Atherosclerosis* 2008; 201: 236-47.
 41. VAN WIJK DF, BOEKHOLDT SM, WAREHAM NJ *et al.*: C-reactive protein, fatal and non-fatal coronary artery disease, stroke, and peripheral artery disease in the prospective EPIC-Norfolk cohort study. *Arterioscler Thromb Vasc Biol* 2013; 33: 2888-94.
 42. VERMA S, KULISZEWSKI MA, LI SH *et al.*: C-reactive protein attenuates endothelial progenitor cell survival, differentiation, and function: further evidence of a mechanistic link between C-reactive protein and cardiovascular disease. *Circulation* 2004; 109: 2058-67.
 43. FUJII H, LI SH, SZMITKO PE, FEDAK PW, VERMA S: C-reactive protein alters antioxidant defenses and promotes apoptosis in endothelial progenitor cells. *Arterioscler Thromb Vasc Biol* 2006; 26: 2476-82.
 44. AKIRA S, UEMATSU S, TAKEUCHI O: Pathogen recognition and innate immunity. *Cell* 2006; 124: 783-801.
 45. GOH FG, MIDWOOD KS: Intrinsic danger: activation of Toll-like receptors in rheumatoid arthritis. *Rheumatology (Oxford)* 2012; 51: 7-23.
 46. RAICEVIC G, ROUAS R, NAJAR M *et al.*: Inflammation modifies the pattern and the function of Toll-like receptors expressed by human mesenchymal stromal cells. *Hum Immunol* 2010; 71: 235-44.
 47. ZHOU PH, LIU SQ, PENG H: The effect of hyaluronic acid on IL-1beta-induced chondrocyte apoptosis in a rat model of osteoarthritis. *J Orthop Res* 2008; 26: 1643-8.
 48. ITO C, KUSANO E, AKIMOTO T *et al.*: Cilostazol enhances IL-1beta-induced NO production and apoptosis in rat vascular smooth muscle via PKA-dependent pathway. *Cell Signal* 2002; 14: 625-32.
 49. CASTEJON R, JIMENEZ-ORTIZ C, VALERO-GONZALEZ S, ROSADO S, MELLOR S, YEBRA-BANGO M: Decreased circulating endothelial progenitor cells as an early risk factor of subclinical atherosclerosis in systemic lupus erythematosus. *Rheumatology (Oxford)* 2014; 53: 631-8.
 50. LAURENT S, BOUTOUYRIE P, ASMAR R *et al.*: Aortic stiffness is an independent predictor of all-cause and cardiovascular mortality in hypertensive patients. *Hypertension* 2001; 37: 1236-41.