Accumulation of VEGFR-2+/CD133+ cells and decreased number and impaired functionality of CD34+/VEGFR-2+ cells in patients with SLE

Petra Ebner1, Frauke Picard1, Jutta Richter2, Eleonore Darrelmann1, Matthias Schneider2, Bodo-Eckehard Strauer1 and Michael Brehm1

Abstract

Objective. Inflammation and atherosclerosis are the major causes of cardiovascular disease (CVD) in SLE. Both traditional and disease-specific risk factors contribute to the formation of endothelial dysfunction. Endothelial progenitor cells (EPCs) have the ability to restore endothelial integrity. The aim of this study was to determine whether the number and function of EPCs are altered in SLE.

Methods. Nineteen patients with SLE and 19 controls were analysed. VEGF receptor-2 (VEGFR-2)+/CD133+ and CD34+/VEGFR-2+ cells were quantified by flow cytometry. EPC differentiation was measured by Dil-acLDL/Lectin I staining. Furthermore, apoptosis, proliferation capacity, migration capacity and clonogenic ability of EPCs were determined.

Results. VEGFR-2+/CD133+ cells were enhanced in SLE [215 (37) vs 122 (11) cells/1×10⁶ lymphocytes; \(P=0.029\)], whereas the number [106 (13) vs 215 (27) cells/1×10⁶ lymphocytes; \(P=0.002\)] and the proliferation rate [96% (6%) vs 143% (19%); \(P=0.008\)] of CD34+/VEGFR-2+ cells were decreased compared with controls. Additionally, EPCs in SLE showed an increased apoptosis [7% (1.4%) vs 3% (0.4%); \(P=0.004\)], an impaired differentiation [36 (5) vs 121 (20) cells/mm²; \(P<0.001\)] and a reduced migratory capacity [116% (4%) vs 139% (4%); \(P=0.001\)].

Conclusions. Our results suggest that the mobilization of progenitor cells is unaffected in SLE, but the diminished number and the altered functionality of circulating CD34+/VEGFR-2+ cells reduce the ability to repair vascular damage and thus may trigger the development of atherosclerosis in SLE.

Key words: Systemic lupus erythematosus, Endothelial progenitor cells, Early endothelial progenitor cells, Neovascularization, Angiogenesis.

Introduction

SLE is an autoimmune disease characterized by chronic inflammation and a strong predisposition for cardiovascular disease (CVD) [1]. Accelerated atherosclerosis is a major problem in SLE and is one of the main causes of death [2]. The incidence of myocardial infarction in women with SLE aged 35–44 years was 50-fold higher than in women of similar ages from a population-based sample [3]. The pathophysiology of SLE-associated atherosclerosis is not completely clarified, but may depend on a profound endothelium dysfunction [4]. It could be demonstrated that a combination of traditional risk factors (such as hypertension, dyslipidaemia and diabetes) and disease-specific factors are responsible for the high risk of CVD in SLE [5].

Disease-specific autoantibodies like AECA, aPL and anti-dsDNA antibody (anti-dsDNA) affect endothelial cells directly or indirectly, causing inflammatory vascular damage [6], endothelial cell apoptosis [7] and increase the risk of arterial and venous thrombosis [8].

Mobilization of progenitor cells from bone marrow and their recruitment into injured tissue are important steps for regeneration and healing. Endothelial progenitor cells...
(EPCs) are a heterogeneous group that exist in a variety of developmental stages ranging from haemangioblasts to fully differentiated endothelial cells. The immature EPCs can be characterized by the expression of CD133, CD34 and VEGF receptor-2 (VEGFR-2) (KDR). During differentiation from immature to mature EPCs CD133 expression is lost. The mature EPCs begin to express vascular endothelial (VE)-cadherin and von Willebrand factor, in addition to CD34 and VEGFR-2 [9, 10]. Proangiogenic factors such as VEGF can mobilize EPCs and may potentiate their recruitment to the site of endothelial injury. Once incorporated, the EPC develops into a mature endothelial cell [11].

The integrity of the endothelium depends on the balance between endothelial turnover/vascular injury and repair, which involves the recruitment of EPCs in circulating blood [12]. All these findings of EPC biology suggest a protective role of EPCs during the progression of atherosclerotic disease and may reflect the endogenous vascular repair capacity [13]. Consequently, the amount of circulating EPCs can serve as a biomarker, because their amount in peripheral blood is a strong surrogate marker for vascular dysfunction and cardiovascular risk [14]. Several reports have shown that reduced levels and an impaired function of EPCs inversely correlate with risk factors of coronary artery disease, endothelial function and the risk for CVD [4, 15].

The above mentioned suggests that on the one hand, EPCs have a protective role during the progression of atherosclerosis based on their capacity for vascular repair. On the other hand, however, it was demonstrated that patients with SLE have an enhanced cardiovascular risk and a stronger precondition for atherosclerotic disease. Taking these findings together, we came to the hypothesis that a reduced amount and/or an impaired function of EPCs were responsible for this condition.

In conclusion, the objective of our study was to determine if the number and function of EPCs were impaired in patients with SLE.

**Patients and methods**

**Study population**

Our study population consists of 19 female patients with SLE, who were selected from the routine visit to our outpatient clinic from July 2005 to November 2007. All patients were white Caucasians and they fulfilled the 1997 revised criteria of the ACR. The average duration of the disease was 13 years. In all patients the disease was in an inactive stage. Exclusion criteria were cancer, pregnancy, diabetes mellitus, impaired renal function and presence of CVD. The control group comprised 19 healthy, age-matched Caucasian women. Demographic characteristics, laboratory measurement and information about medications are shown in Table 1. The study was approved by the local Ethics Committee of the Heinrich-Heine University in Düsseldorf and informed consent was obtained from all participants.

**Isolation and cultivation of blood-derived progenitor cells**

Thirty millilitres of peripheral venous blood was taken with BD Vacutainer CPT™ System containing a citrate anti-coagulant with Ficoll Hypaque density fluid and a polyester gel barrier (BD Biosciences, Franklin Lakes, NJ, USA). The samples were centrifuged for 20 min at 400 g at room temperature. The mononuclear cells (MNCs) were resuspended into the plasma by inverting the tube, and the entire content above the gel was transferred into a separate tube. After centrifugation, the MNCs were treated with ammonium chloride for 10 min to accomplish the lysis of all red blood cells. After washing twice with phosphate-buffered saline (PBS), MNCs were resuspended in 1 ml PBS. The cell number was determined in a Neubauer chamber.

**Flow cytometry analysis**

Two-colour flow cytometry assays were performed using an EPICS XL flow cytometer (Beckman Coulter/Immunotech, Roissy CDG, France) and Expo 32 data acquisition software (Beckman Coulter/Immunotech). For red blood cell lysis, peripheral blood samples were diluted in 15 ml bicarbonate-buffered ammonium chloride solution (0.15 M NH₄Cl, 0.01 M NaHCO₃ and 1.0 mM EDTA) for 15 min at room temperature. The cells were centrifuged and resuspended in 500 μl PBS supplemented with 10% fetal calf serum (FCS), pH 7.2 (lyse-no-wash, technique).

Briefly, 100 μl of white blood cell samples were incubated with anti-VEGFR-2 (purified; Reliatech, Braunschweig, Germany) for 20 min at 4°C. After incubation, cells were washed with PBS/20% FCS. The purified anti-VEGFR-2/KDR-antibody was incubated with FITC-conjugated anti-mouse IgG1 or phycoerythrin (PE)-conjugated anti-mouse IgG1 (BD Pharmingen, Franklin Lakes, NJ, USA) for 20 min at 4°C. Cells were washed with 1 ml PBS/10% FCS and incubated with FITC-conjugated anti-CD34 (BD Biosciences) or PE-conjugated anti-CD133/1 (Miltenyi Biotec, Bergisch Gladbach, Germany) for 20 min at 4°C. Subsequently, cells were washed with 1 ml PBS/10% FCS and fixed with 500 μl 1% paraformaldehyde for 10 min at 4°C. Finally, cells were washed with 1 ml PBS/10% FCS and resuspended in 500 μl PBS/10% FCS. Background levels of staining were measured using isotype controls (BD Biosciences). Stained cells were determined and compared with appropriate negative controls. Positive staining was defined as being greater than non-specific background staining. All samples were measured in duplicate.

The lymphocyte fraction was identified on a forward/side scatter, and a minimum of 1 × 10⁶ lymphocytes were counted. Cells positive for VEGFR-2/CD133 within the lymphocyte fraction were characterized as early EPCs, whereas cells positive for CD34/VEGFR-2 within the lymphocyte fraction were characterized as mature EPCs. Results were expressed as the number of EPCs per 1 × 10⁶ lymphocytes.
EPC number and differentiation

Cells (1 × 10⁶) were seeded onto a fibronectin-coated well of a four-well tissue plate in endothelial basal medium MV2 (EBM MV2; Promocell, Heidelberg, Germany) supplemented with EBM-MV2 SingleQuots. Culture medium was replaced on day 3. After 7 days in culture, cells were incubated with 2.5 \( \text{mg/ml} \) DiI-acLDL (Biomedical Technologies, Birkenfeld, Germany) at 37°C for 1 h. Cells were subsequently fixed with 1% paraformaldehyde for 10 min at 4°C and counterstained with Ulex Europaeus Lectin conjugated to FITC (UEA-1-FITC; Biomeda, Foster City, CA, USA) for 3 h. Cells were characterized as annexin-V positive cells and determined by fluorescence microscope (Nikon). Forty HPFs were analysed by two independent investigators. The percentage of apoptotic cells was calculated among the total cells per HPF.

Apoptosis of EPCs

In the early stages of apoptosis, changes occur at the cell surface. One of these plasma membrane alterations is the translocation of phosphatidylserine (PS) from the inner part of the plasma membrane to the outer layer, by which PS becomes exposed at the external surface of the cell. Annexin-V-Fluos binds in a Ca²⁺-dependent manner to negatively charged phospholipid surfaces and shows high specificity to phosphatidylserine, indicating an early stage of cell apoptosis. Propidium iodide (PI) was required to discriminate apoptotic cells from necrotic cells.

Cells (1 × 10⁶) were seeded onto a fibronectin-coated well of a four-well tissue plate in endothelial basal medium MV2 (EBM MV2; Promocell), supplemented with EBM-MV2 SingleQuots. Culture medium was replaced on day 3. After 7 days in culture, cells were incubated with Annexin-V-Fluos labelling reagent and PI solution (Annexin-V-FLUOS Staining Kit; Roche, Mannheim, Germany) for 15 min at room temperature. Apoptotic cells were characterized as annexin-V positive cells and were determined by fluorescence microscope (Nikon). Forty HPFs were analysed by two independent investigators. The percentage of apoptotic cells was calculated among the total cells per HPF.

### Table 1 Characteristics of patients and controls

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>SLE, n = 19</th>
<th>Controls, n = 19</th>
<th>P-value</th>
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<tbody>
<tr>
<td><strong>Demographics</strong></td>
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<tr>
<td>Gender, women/men</td>
<td>19/0</td>
<td>19/0</td>
<td>–</td>
</tr>
<tr>
<td>Age, years</td>
<td>36 (6)</td>
<td>37 (8)</td>
<td>0.744</td>
</tr>
<tr>
<td><strong>Cardiovascular risk factors</strong></td>
<td></td>
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<tr>
<td>Smoking, %</td>
<td>25</td>
<td>31.25</td>
<td>0.527</td>
</tr>
<tr>
<td>Hyperlipidaemia, %</td>
<td>12.5</td>
<td>18.75</td>
<td>0.564</td>
</tr>
<tr>
<td>Diabetes mellitus, %</td>
<td>0</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>56.3</td>
<td>6,25</td>
<td>0.02</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>66 (15)</td>
<td>65 (11)</td>
<td>0.909</td>
</tr>
<tr>
<td><strong>Clinic and labour</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP, mg/dl</td>
<td>&lt;0.3</td>
<td>&lt;0.3</td>
<td>1.0</td>
</tr>
<tr>
<td>Leucocytes × 10⁰⁶ /μl</td>
<td>7.1 (2.7)</td>
<td>7.3 (1.9)</td>
<td>0.557</td>
</tr>
<tr>
<td>Serum C3, mg/dl</td>
<td>86.6 (19.6)</td>
<td>115.8 (17.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum C4, mg/dl</td>
<td>15.0 (6.5)</td>
<td>23.7 (8.7)</td>
<td>0.001</td>
</tr>
<tr>
<td>Cholesterol, mg/dl</td>
<td>175.5 (45.5)</td>
<td>188.9 (49.2)</td>
<td>0.654</td>
</tr>
<tr>
<td>LDL, mg/dl</td>
<td>124.4 (22.5)</td>
<td>141.0 (47.5)</td>
<td>0.724</td>
</tr>
<tr>
<td>HDL, mg/dl</td>
<td>76.4 (29.5)</td>
<td>67.6 (17.6)</td>
<td>0.118</td>
</tr>
<tr>
<td>Creatinine, mg/dl</td>
<td>1.3 (1.4)</td>
<td>0.7 (0.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Positive dsDNA antibody, U/ml</td>
<td>69.4 (118.2)</td>
<td>&lt;5</td>
<td>0.424</td>
</tr>
<tr>
<td>Positive phospholipids antibody, IU/ml</td>
<td>&lt;8</td>
<td>&lt;8</td>
<td>0.597</td>
</tr>
<tr>
<td>Disease duration, years</td>
<td>13 (8)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Clinically inactive disease, months</td>
<td>40 (26)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Medication, %</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prednisolone ≤ 7.5 mg/day</td>
<td>75</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>Prednisolone &gt; 10 mg/day</td>
<td>25</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>AZA</td>
<td>43.75</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>HCQ</td>
<td>62.5</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>Anti-hypertensives</td>
<td>56.25</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>Prednisolone + AZA</td>
<td>31.25</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>AZA/HCQ</td>
<td>25</td>
<td>0</td>
<td>–</td>
</tr>
</tbody>
</table>

Data are presented as mean (S.E.M.) unless otherwise mentioned.
Proliferative capacity of EPCs

The proliferation capacity of endothelial progenitor cells was measured by using the cell proliferation BrdU-ELISA kit (Roche). Therefore, the MNCs were cultivated in EBM-MV2 medium (Promocell) for 7 days. Subsequently, the EPCs were trypsinized and resuspended at a density of $1 \times 10^5$ cells/ml in EBM-MV2 medium with and without 100 ng VEGF/ml and placed on a 96-well plate (100 µl per well). After 24 h, cells were incubated with 10 µM BrdU for 2 h at 37°C/5% CO₂. Then the cells were fixed by adding FixDenat and by incubating in this solution for 30 min at 20°C. After washing, the cells were treated with anti-BrdU for 90 min. Then the antibody conjugate was removed by flicking off and rinsing. Finally, the cells were incubated with substrate solution before the rate of BrdU uptake (proliferation) was determined by measuring the absorbance at 450 nm wavelength using a scanning multiwell spectrophotometer (ELISA reader). The proliferation rate was calculated as the percentage of proliferation in a medium containing VEGF compared with the proliferation in a medium without VEGF.

Migratory activity of EPCs

About $5 \times 10^5$ MNCs were seeded onto a fibronectin-coated culture plate and cultivated in a EBM-MV2 medium (Promocell), supplemented with EBM-MV2 SingleQuots. Culture medium was replaced on day 3. After 7 days in culture, adherent cells were trypsinized and resuspended at a density of $3 \times 10^5$ cells/ml in EBM-MV2 medium (without supplements and growth factors, Promocell), supplemented with 0.1% BSA. The cells were then placed in the top compartment of a Boyden chamber. The bottom compartment contained EBM-MV2 medium (only supplemented with 0.1% BSA) with or without 100 ng/ml VEGF. Cells migrated for 24 h at 37°C in humidified atmosphere. The migrated cells were stained with Diff-Quick Staining Kit (Dade Behring, Marburg, Germany) and then counted by two independent investigators. The migratory activity was calculated as the percentage of migration increase on VEGF compared with the spontaneous migration on medium only.

Colony-forming unit assay

MNCs per millilitre ($1 \times 10^5$) were seeded in Methocult GF H4434 (Stemcell Technologies, Köln, Germany). Culture dishes were seeded with 1 ml cell suspension and then incubated at 37°C in 5% CO₂. After 14 days of incubation, colony-forming unit (CFU) colonies [CFU total colony count and CFU-GM (granulocyte–macrophage colonies)] were counted using a phase contrast microscope by two independent investigators.

Statistics

Statistical analysis was performed using SPSS (version 12.0). The significance of differences between categorical variables was determined by chi-square analysis. Student’s t-test was used to compare continuous variables. For not normally distributed data, the Mann-Whitney U-test was used. Data were expressed as the mean (S.E.M.). P-values <0.05 were considered as statistically significant.

Results

Circulating EPC levels in SLE

The cardiovascular risk factors with regard to smoking, dyslipidaemia, diabetes and hyperlipidaemia did not differ significantly between the patient group and the healthy controls (Table 1).

In patients with SLE, the level of CD34+/VEGFR-2+ positive cells (mature EPCs) was significantly reduced [106 (13) vs 215 (27) cells/1 $\times 10^6$ lymphocytes; $P = 0.002$; Fig. 1A] when compared with healthy persons.

Furthermore, we could demonstrate that the level of more immature population of EPCs (VEGFR-2+/CD133+) was significantly increased in patients with SLE compared with healthy women [215 (37) vs 122 (11) cells/1 $\times 10^6$ lymphocytes; $P = 0.029$; Fig. 1B].

Decreased differentiation of EPCs in SLE

Within the MNC fraction, immature progenitor cells are able to differentiate in vitro into mature EPC demonstrating their characteristic spindle-like shape. By immunohistological staining, these cells show characteristic features of EPCs. They are stained double positive for acLDL and UEA-lectin-1. Since the level of circulating mature EPCs was reduced in SLE patients, we analysed the differentiation potential of MNCs to determine whether these cells are capable of differentiating into mature EPCs. Counting the differentiated EPCs, detected by the uptake of acLDL and the UEA-lectin 1 binding, in the four-well plate revealed a 4-fold decrease of mature EPCs in vitro after 7 days of cultivation of MNCs from SLE patients compared with healthy women [36 (5) vs 121 (20) cells/mm²; $P < 0.001$; Fig. 2].

Increased apoptosis of EPCs from SLE patients

The apoptotic rate of progenitor cells was determined with fluorescence microscope. The percentage of apoptotic cultured EPCs, defined as positive for annexin-V was 4-fold higher in patients with SLE compared with healthy persons [7% (1.4%) vs 3% (0.4%); $P = 0.004$; Fig. 3B].

Decreased proliferation rate of cultivated EPCs

In order to evaluate if the decreased number of EPCs was a consequence of an impaired proliferation rate, we assessed these by the incorporation of BrdU. Our data, obtained by ELISA reader, revealed a significantly diminished proliferation rate of cultured EPCs in patients with SLE [96% (6%) vs 143% (19%); $P = 0.008$] compared with the control group (Fig. 3B).

Migration capacity of circulating EPCs from SLE patients

Because of the importance for neovascularization and the repair of the endothelial cell layer, we analysed the
capacity of cultured EPCs to migrate by measuring their migratory response to VEGF, which represents a chemotactant for EPCs. Our assay indicates that the cultivated EPCs of patients with SLE showed a significantly impaired migratory capacity towards VEGF compared with healthy controls [116% (4%) vs 139% (4%); \( P = 0.001 \); Fig. 4A].

**Functional capacity of EPC for colony formation**

The functional capacity of progenitor cells in the peripheral blood was determined by measuring the number of CFUs. The MNCs from SLE patients showed a significant decrease of CFU-GM [5 (1) vs 23 (5) colonies/ml; \( P = 0.003 \)] and a small but not significant decrease of total colony counts [40 (8) vs 38 (6) colonies/ml; \( P = \text{NS} \)] compared with controls (Fig. 4B).

**Discussion**

Our data support the hypothesis that EPCs are diminished and dysfunctional in patients with SLE. We could demonstrate that the level of circulating mature EPCs (CD34\(^+\)/VEGFR-2\(^+\)) in the peripheral blood of SLE patients is significantly reduced, whereas the level of early immature EPCs (VEGFR-2\(^+\)/CD133\(^+\)) is increased. Furthermore, the proliferation and migration capacity of cultivated EPC were diminished, whereas the apoptotic rate was increased. The observed decreased amount of mature EPCs corresponds with our expectations, because these findings support our hypothesis that a reduced amount of EPCs were responsible for the higher cardiovascular risk in patients with SLE. Other studies also reported a decreased level of circulating EPC [16, 17], except by Grisar et al. [18], who reported a normal level of circulating EPCs in the peripheral blood...
of patients with SLE. An explanation for this discrepancy could be methodological differences, due to the fact that Grisar and his co-workers enumerated EPCs by the surface-marker combination CD34⁺/CD133⁺/VEGF-R2⁺. This combination captures only a small proportion of the EPC population because the CD133-marker is rapidly down-regulated after release into the peripheral blood and replaced by others like CD31 and von Willebrand factor [19]. In contrast to Grisar, we applied the commonly used combination of CD34⁺/VEGFR-2⁺, which detects most of the EPC subpopulations including the more mature EPCs, which no longer express CD133 [20].

Patients with SLE have an increased risk of developing premature CVD [21]. Certain traditional risk factors, i.e. hypertension, diabetes mellitus and age, partially account for the development of CVD in patients with SLE [22, 23]. However, these risk factors do not fully explain the increase in accelerated atherosclerosis in SLE.

The pathogenesis of atherosclerosis in these patients also involves disease-related factors including metabolic abnormalities such as premature menopause, elevated very low-density lipoprotein (VLDL) cholesterol, triglycerides [24], sedentary lifestyle, obesity, chronic inflammation and aPL antibodies [25, 26].
The exposure to both traditional and disease-specific risk factors leads to endothelial injury and endothelial apoptosis [27], with the result of endothelial dysfunction and inflammatory response, which are the initial steps to atherogenesis and the progression of atherosclerosis [28].

For limiting atherosclerosis and reducing the cardiovascular events, endothelial repair and regeneration are important [29]. In this context, the balance between endothelial damage and reparative mechanism is critical. Recent reports have shown that reduced levels and impaired function of EPCs inversely correlate with risk factors of coronary artery disease, endothelial function [4] and the risk of CVD.

EPCs are a group of cells that exist in a variety of developmental stages ranging from haemangioblasts to fully differentiated endothelial cells that support the re-endothelization of injured arteries by replacing the dysfunctional endothelial cells [30]. Their mobilization from bone marrow into the circulation is a complex process, regulated by a variety of factors, for example, VEGF, SDF-1 or NO [10, 31].

The circulating progenitor cells home to sites of intimal damage [32], where they can actively repair the endothelial layer through incorporation and differentiation into endothelial cells (vasculogenesis). SLE is often associated with chronic vascular inflammation [33], which is likely attributable to the consequence of endothelial dysfunction and thus an ideal condition for the development of atherosclerotic plaque [34].

A study from Torsney et al. [35] suggested an increased consumption of mature EPCs at the sites of vascular injury. This report demonstrated that only mature EPCs accumulate in atherosclerotic lesions, which could be possible reason for the observed low amount of CD34+/VEGFR-2+ EPCs in the circulation of patients with SLE.

Like Pyrovolaki et al. [36] described an increased apoptosis rate in CD34+ cells expressing CD40 in patients with SLE, we also found an increased apoptosis of CD34+/VEGFR-2+ cells. Furthermore, we demonstrated a limited proliferation rate of these cells. We assume that these results are the main explanation for the diminished level of mature EPCs.

Furthermore, it cannot be ruled out that the medication is responsible for the observed decrease in mature EPCs. It was demonstrated that prednisolone doses >10 mg/day were associated with hypercholesterolaemia, increased total cholesterol, VLDL cholesterol and LDL cholesterol [37], which could be a negative impact on the amount of EPCs [38]. Indeed, Frostegård et al. [39] reported that low-dose prednisolone did not influence atherosclerosis in patients with RA and it is presumed that this will be similar in SLE. Most of the examined patients received doses of prednisolone <10 mg/day (only one patient received 12.5 mg/day; Table 2), thus, the effect of prednisolone with regard to lowering of mature EPCs in patients with SLE is probably negligible.

The role of AZA deserves additional consideration. AZA is normally used as an immunosuppressive drug because of its anti-proliferative qualities. The study of Weigl et al. [40] indicated that this drug had a toxic effect on endothelial cells. It cannot be ruled out that AZA has a similar effect on CD34+/VEGFR-2+ cells. This aspect needs further investigation with focus on the effect of AZA on EPCs.

Furthermore, to our knowledge, this is the first study which documents that the VEGFR-2+/CD133− cell population in SLE patients is 2-fold higher than in the control group. In contrast to our findings, Moonen and colleagues [41] observed a reduced level of CD133+ cells. Contrary to our method, Moonen used a restrictive combination of CD34+/VEGFR-2− for quantification. This detects only the subpopulations positive for CD34+. Our combination of VEGFR-2+/CD133+ is more variable because it includes both CD34+ and CD34− subtypes of early EPCs. Studies reported that a majority of peripherally derived EPCs did not only originate from CD34+ subpopulations but also from populations that are negative for the CD34 marker [42]. Friedrich et al. demonstrated that this CD34− EPC subpopulation has potent vasoregenerative capacities [43].

EPCs are characterized by their co-expression of progenitor (CD34 or CD133) and endothelial lineage (VEGFR-2) surface markers. Early EPCs, which are positive for CD34+/CD133+/VEGFR-2+, are found in bone marrow [20]. Usually only a small proportion of CD133+ cells is found in the systemic circulation.

Chronic or recurrent inflammation and chronic exposure to inflammatory factors in patients with SLE could lead to endothelial damages and increased endothelial turnover. It is assumed that the above-mentioned factors cause an increased requirement of an endothelial renewal with the

<table>
<thead>
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<th>Table 2 Overview of study results</th>
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<tr>
<td><strong>Assay</strong></td>
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<tr>
<td>CD34+/VEGFR-2+, cells/1 × 10⁶ lymphocytes</td>
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<tr>
<td>VEGFR-2+/CD133+, cells/1 × 10⁶ lymphocytes</td>
</tr>
<tr>
<td>EPC differentiation, cells/mm²</td>
</tr>
<tr>
<td>Proliferation, %</td>
</tr>
<tr>
<td>Apoptosis, %</td>
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<tr>
<td>Migration, %</td>
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<tr>
<td>CFU total, cells/1 × 10⁵ MNCs</td>
</tr>
<tr>
<td>CFU-GM, cells/1 × 10⁵ MNCs</td>
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</table>

Data are presented as mean (s.e.m.).
consequence of an increased mobilization of VEGFR-2+/CD133+ cells into the circulation. Because of this accumulation of early EPC, it is unlikely that an impaired mobilization of progenitor cells is accountable for the observed decrease of mature EPCs.

With regard to the high number of premature, early EPCs in the circulation of SLE patients, it should be expected that the number of mature EPCs is similarly high. However, in contrast to this expectation, the mature EPCs were depleted in patients with SLE.

Our data showed an increased apoptosis rate and a limited proliferation rate of mature EPCs, which could be a possible explanation for the observed low amount of CD34+/VEGFR-2+ cells. Furthermore, the results of our differentiation assay revealed that the progenitor cells of patients with SLE have a 4-fold lower capacity to differentiate into mature EPCs compared with controls.

Of course, this effect is partially explainable by the observed 2-fold higher apoptosis rate and the limited proliferation rate of mature EPCs in SLE, but in view of the high amount of immature EPCs in the circulation of patients with SLE, there must be other explanations for the diminished level of mature EPCs.

One further possibility for the decreased number of mature EPCs could be an impaired down-regulation of the CD133 marker. Once in circulation, CD133 is rapidly down-regulated and the progenitor cells begin the process of differentiation to mature EPCs that are still positive for CD34 and VEGFR-2 [44] and endothelial cells. An important step in this transformation process seems to be the loss of the surface marker CD133 and a parallel or subsequent expression of von Willebrand factor in combination with the appearance of other endothelial characteristics. However, the underlying mechanism and the time point at which the EPCs begin to lose CD133 during their transmigration from bone marrow or later during their circulation are unclear [9].

The impaired CD133 down-regulation prevents the differentiation into the mature EPC phenotype and the progenitor cell is locked in its immature state [30], a fact that may lead on the one hand to an enrichment of early EPCs in the circulation and on the other hand to a reduced level of mature EPCs in SLE.

Additionally, the cultivated EPCs of SLE patients showed a limited response to VEGF. Studies indicate that VEGF is important for the differentiation, proliferation and development of bone marrow-derived EPCs to endothelial-like cells [45, 46].

Naturally, it is also conceivable that an elevated proliferation rate of the VEGFR-2+/CD133+ cells accounts for their accumulation in patients with SLE and that further studies, with focus on the VEGFR-2+/CD133+ cells, are needed to reveal the mechanisms and factors for the observed enrichment.

In conclusion, we were able to reveal that VEGFR-2+/CD133+ cells accumulate in the circulation of patients with SLE, which made us presume that the mobilization of progenitor cells is not impaired. Unfortunately, the immature EPCs fail to cause a proangiogenic effect, because these cells possess none or few vaso-regenerative capacities. Mainly, the mature EPCs have the ability to reduce lesion progression, improve endothelial function and prevent atherosclerosis, but our data display that these cells are depleted in patients with SLE. Additionally, the mature EPCs of patients with SLE exhibit an affected functionality because of an increased apoptosis, a decreased proliferation and a limited response against VEGF. It must be assumed that these points are responsible for the high CVD risk observed in women with SLE.

Further studies could address the question: Which mechanisms and pathways account for the impaired differentiation to mature EPC and which effects are necessary to extend the pool of mature EPCs and to improve their function?

Rheumatology key messages

- Impaired number and functionality of CD34+/VEGFR-2+ cells seems to be accountable for atherosclerosis in SLE.
- Mobilization of EPCs seems to be unaffected in SLE.

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