

# Differentiation of Bone Marrow–Derived Endothelial Progenitor Cells Is Shifted into a Proinflammatory Phenotype by Hyperglycemia

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Bone marrow (BM)-derived endothelial progenitor cells (EPC) contribute to vascular maintenance by participating in angiogenesis, re-endothelialization, and remodeling. Myeloid progenitor cells in the BM are functionally and quantitatively an important precursor pool for cells that contribute to these processes. However, these precursor pools in the BM also give rise to important effector cells of the innate immune system, such as macrophages and dendritic cells. We hypothesized that the disturbed repair responses that are being observed in diabetes mellitus are also related to an effect on functional and differentiation characteristics at the level of this bone marrow precursor pool. Indeed, we observed that bone marrow differentiation cultures for EPC, macrophages (Mph), or dendritic cells (DC) from hyperglycemic BM yielded 40% fewer EPC and 50% more Mph compared with control BM. These changes were directly related to the hemoglobin A<sub>1c</sub> levels of the donor mice. BM-derived DC numbers were not affected by hyperglycemia. The composition of the BM was not altered; in particular, the numbers of CD31<sup>+</sup>/Ly6C<sup>+</sup> cells, which serve as common progenitors for EPC, Mph, and DC, were unaffected. In addition, BM-derived EPC from hyperglycemic mice were less angiogenic and more proinflammatory in regards to endocytosis, T-cell activation, and interleukin 12 production. HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase inhibition by statin supplementation of the culture medium counteracted these hyperglycemia-induced changes. Our study results show that hyperglycemia alters the differentiation fate of BM precursor cells, reducing the potential to generate vascular regenerative cells and favoring the development of proinflammatory cells.

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## INTRODUCTION

Loss of endothelial integrity and an impaired capacity for ischemia-induced neovascularization leads to ischemic vascular disease in diabetes (1–3). A recently identified risk factor for these vascular complications is dysfunction of bone marrow (BM)-derived endothelial progenitor cells (EPC). Under normal circumstances, EPC contribute to vascu-

lar homeostasis by replacing apoptotic or lost endothelial cells (4,5). In addition, EPC can be home to sites of denudation (6), ischemia, or elevated shear stress to stimulate neovascularization or arteriogenesis (7,8). In patients with type 1 (9) and type 2 diabetes (10) and in diabetic animals (11–13), the number of circulating EPC is decreased, and functional parameters such as adhesion, migration,

and the paracrine secretion of proangiogenic factors are impaired, likely as a consequence of hyperglycemia. Moreover, a recent study showed that the number of circulating EPC inversely correlates with the severity of peripheral vascular complications of patients with type 2 diabetes, further supporting a role for EPC dysfunction in the pathogenesis of ischemic vascular disease (14). In accordance with these findings, EPC are increasingly recognized as a potential therapeutic target for the prevention of ischemic vascular disease. However, the molecular mechanisms underlying EPC dysfunction in diabetes are complex and may include reduced cell survival (11,15) due to an increased sensitivity to oxidative stress (16), or activation of a

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p53-dependent pathway that leads to cellular senescence (16,17). Human EPC can be derived from CD34<sup>+</sup>, CD34<sup>+</sup>, or CD34<sup>low</sup> cells or can be cultured from BM aspirates and peripheral blood CD14<sup>+</sup> mononuclear cell fractions (18–21).

CD14<sup>+</sup> cells have long been known to play a critical, paracrine role in neovascularization (22), and it is highly likely that “early outgrowth” EPC derived from CD14<sup>+</sup> cells directly reflect the proangiogenic phenotype of monocytes that have entered ischemic tissue that is rich in vascular endothelial growth factor (23,24). We have previously demonstrated that in mouse BM these angiogenic EPC develop from an immature, CD31<sup>+</sup>/Ly-6C<sup>+</sup> myeloid progenitor fraction (25). Given the proper differentiation factors, however, these BM precursor pools also give rise to important effector cells of the innate immune system such as macrophages (Mph) and dendritic cells (DC) (26,27), suggesting a common myeloid progenitor pool for EPC, Mph, granulocytes, monocytes, and DC. Because diabetes is increasingly being recognized to be a chronic systemic proinflammatory state (28,29), we hypothesized that the disturbed vascular repair in diabetes is related to an effect of hyperglycemia on the differentiation fate of myeloid BM precursors. We demonstrated that hyperglycemia indeed skews the BM potential from generating proangiogenic early EPC to the development of proinflammatory cells. Our study results may not only provide new insight into the pathogenesis of vascular complications in diabetes but also be relevant for the use of BM cells as a source for therapeutic neovascularization.

## MATERIALS AND METHODS

### Animals

Moderate to severe hyperglycemia was induced in 6-wk-old C57BL/6J and FVB/N mice (Harlan, Horst, the Netherlands) by intraperitoneal (IP) injections of streptozotocin (STZ), 80 mg/kg body

weight (Sigma-Aldrich, St. Louis, MO, USA) in 0.05 mol/L Na-citrate buffer, pH 4.5, on two consecutive d. Blood glucose levels were examined with a glucose-oxidase technique (OneTouch UltraSystem Lifescan, Milpitas, CA, USA) 1 wk after STZ injection, and mice with blood glucose levels below 20 mmol/L received two additional injections on two consecutive d. When necessary this procedure was repeated a third time. STZ treatment was stopped when blood glucose levels above 20 mmol/L were reached, and glucose levels and total body weight were monitored weekly for a period of 6 wks. Control mice were injected with buffer only at the onset of the experiments. Mice from the STZ treatment groups that showed no elevated blood glucose levels (below 15 mmol/L) were excluded from the study. All procedures involving animal handling were in accordance with national and institutional guidelines of the Erasmus MC, Rotterdam, the Netherlands.

### Glycosylated Hemoglobin

Glycosylated hemoglobin (HbA<sub>1c</sub>) levels were measured by high performance liquid chromatography using a reverse-phase cation exchange column and detected by a dual-wavelength colorimetric (415 and 500 nm) analyzer (ADAMS A1c HA8160; Arkray, Tokyo, Japan).

### Isolation and Differentiation of Murine EPC, DC, and Mph

BM-cell suspensions were prepared by flushing femora and tibiae with RPMI medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Gibco) and antibiotics (penicillin/streptomycin, Gibco). To culture EPC, total BM cell suspensions were plated at a density of  $1.25 \times 10^6$  cells per cm<sup>2</sup> in 24-well plates (Nunc, Rochester, NY, USA) coated with fibronectin (10 µg/mL, Sigma). Cells were cultured for 7 d in M199 medium supplemented with 20% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 0.05 mg/mL bovine pituitary extract (Invitrogen), antibiotics, and

10 units/mL heparin (Leo Pharma BV, Breda, the Netherlands).

For Mph and DC,  $2 \times 10^6$  BM cells were cultured in complete RPMI medium described above and plated on 10-cm Petri dishes (BD Biosciences, Franklin Lakes, NJ, USA). Medium was replaced every 2 d. For DC and Mph cultures, the medium was supplemented with 20 ng/mL recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF) (BioSource, Nivelles, Belgium) and 10 ng/mL recombinant murine M-CSF (PreproTech, Rocky Hill, NJ, USA), respectively. Atorvastatin (Pfizer, New York, NY, USA) was dissolved in ethanol and, in indicated experiments, added to the medium to a final concentration of 0.1 µmol/L. Medium used in the control experiments contained ethanol at a similar final concentration ( $5 \times 10^{-5}\%$ ), as present in the cultures treated with atorvastatin.

### Monoclonal Antibodies, Conjugates, and Flow-Cytometric Analysis

Undiluted culture supernatant of the hybridoma F4/80 was directly used for staining. ER-MP12/CD31 was purified and biotinylated, and ER-MP20/Ly-6C was conjugated to FITC (26,30). Phycoerythrin (PE)-labeled CD11c and biotinylated anti-major histocompatibility complex class II antibodies were purchased from BD Biosciences. Secondary fluorescein isothiocyanate (FITC)- or R-PE-labeled goat antirat IgG antibodies (mouse-absorbed; GαR-FITC or GαR-PE) were purchased from Caltag Laboratories (Burlingame, CA, USA). Biotinylated antibodies were detected with allophycocyanin-conjugated streptavidin (BD Biosciences). For EPC characterization, cells were stained with rhodamine-labeled *Bandeiraea simplicifolia* lectin, 10 µg/mL, (BSL-1; Vector Labs, Burlingame, CA, USA) for 1 h. To measure the uptake of Dil-labeled acetylated low-density lipoprotein (acLDL) (Molecular Probes, Eugene, OR, USA), cells were incubated with acLDL (2.4 µg/mL) at 37°C for 2 h.

Flow cytometric analyses were done on a Calibur flowcytometer (BD Biosciences) using CellQuest software (BD Biosciences).

### Cytokine Detection

For cytokine measurements EPC were incubated with 50 ng/mL lipopolysaccharide (Sigma) overnight at 37°C. Culture supernatants were collected and cells were counted for normalization. Interleukin (IL)-12p40 and IL-12p70 ELISA kits (R&D Systems, Minneapolis, MN, USA) were used according to the manufacturer's protocol.

### Mixed Lymphocyte Reaction Assay

Mixed lymphocyte reaction assays were done with allogeneic T cells from C57BL/6J or FVB/N splenocytes. Cells were incubated with antibodies recognizing CD11b, CD45, and major histocompatibility complex class II and anti-rat IgG microbeads. Naive T cells were obtained by negative selection using a magnetic cell sorter. DC, Mph, and EPC were irradiated sublethally. T cells ( $1.5 \times 10^5$  cells/well) were added to varying concentrations of stimulator cells depending on the desired stimulator-responder cell ratio. Proliferation of T cells was measured after 4 d by uptake of  $^3\text{H}$ -thymidine, 1  $\mu\text{Ci}$ /well (DuPont-NEN, Boston, MA, USA) measured as counts/min.

### In vitro Angiogenesis Assay

Conditioned media were obtained by replacing the medium of 6-d EPC cultures with serum-free endothelial cell basal medium-2 (Clonetics, Walkersville, MD, USA) supplemented with endothelial growth medium 2 single aliquots (without vascular endothelial growth factor and basic fibroblast growth factor) and culturing the cells for an additional 20 h. EPC were counted, and conditioned media were diluted to correct for cell numbers. After 14 h, tube formation by human umbilical vein endothelial cell (HUVEC) was measured by staining the viable cells with Calcein-AM, 5  $\mu\text{g}/\text{mL}$  (Molecular Probes). HUVEC cultures

**Table 1.** Mouse characteristics.<sup>a</sup>

	C57BL/6J Control	C57BL/6J STZ	FVB/N Control	FVB/N STZ
n	18	26	17	23
Blood glucose, mmol/L	6.7 $\pm$ 2.0	24.1 $\pm$ 5.5 <sup>b</sup>	9.1 $\pm$ 1.8	27.8 $\pm$ 4.5 <sup>b</sup>
HbA <sub>1c</sub> , %	3.9 $\pm$ 0.2	7.6 $\pm$ 0.7 <sup>b</sup>	4.1 $\pm$ 0.5	5.9 $\pm$ 0.5 <sup>b</sup>
Weight, g	28.5 $\pm$ 2.2	20.0 $\pm$ 1.2 <sup>b</sup>	30.5 $\pm$ 2.3	22.5 $\pm$ 2.9 <sup>b</sup>
Bone marrow, cells $\times 10^8$	0.8 $\pm$ 0.3	0.8 $\pm$ 0.2	0.7 $\pm$ 0.2	0.9 $\pm$ 0.3

<sup>a</sup>Parameters were measured after 6 wks of STZ-induced hyperglycemia or in littermate control mice matched for age and sex that were injected with buffer. Values represent mean  $\pm$  SD.

<sup>b</sup> $P < 0.05$  versus buffer.

were isolated as described (31) and used at passage 3 or less. For quantification, total tube area was determined using images obtained with an inverted fluorescence microscope and Scion Imaging software (Scion Corporation) and expressed in arbitrary units (AU) of tube length.

### Endocytosis Assay

Uptake of dextran-FITC was determined for 30 min, both at 4°C and 37°C. Subsequently, the cells were washed three times with phosphate-buffered saline/bovine serum albumin 1% and total uptake of FITC label was measured by fluorescence-activated cell-sorting analyses and expressed by the difference in geometric mean that resulted from subtracting the values obtained at 4°C from the values obtained at 37°C.

### Statistical Analysis

Differences between treatment groups were analyzed by use of the Student *t* test. Results are expressed as mean  $\pm$  SD. Linear regression analyses and Pearson correlation were used for comparison of the number of EPC/Mph and HbA<sub>1c</sub>. Probability values of  $P < 0.05$  were considered statistically significant.

## RESULTS

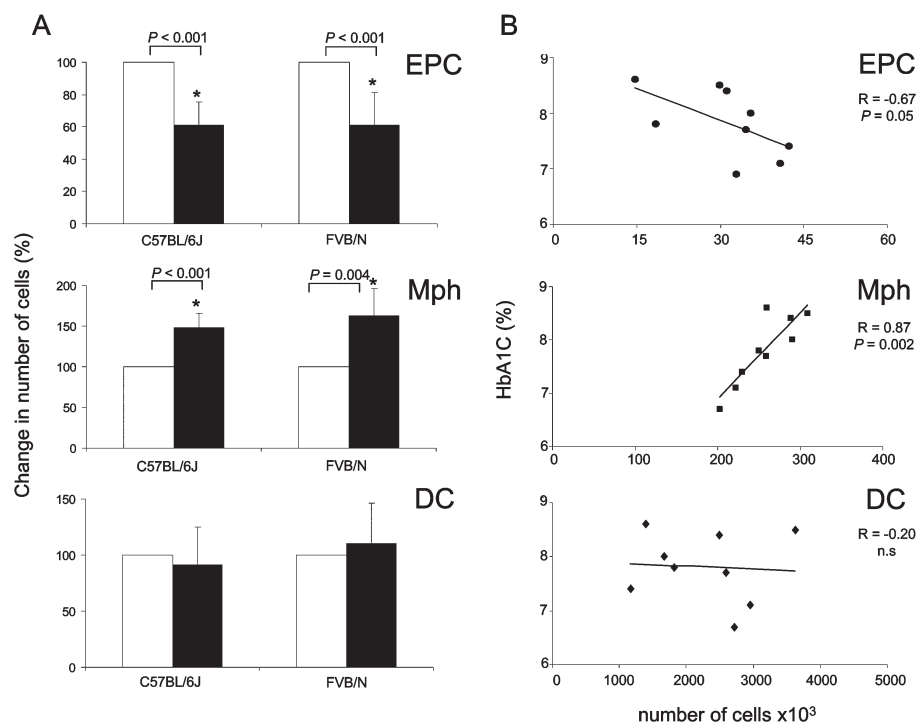
### Hyperglycemia Induction in Mice

In both mouse strains used (C57BL/6J and FVB/N), STZ treatment increased blood glucose levels up to 3.5 fold, an increase associated with increased

HbA<sub>1c</sub> levels (Table 1). Body weight was reduced 25%–30% in hyperglycemic mice of both strains compared with normoglycemic control animals. Total BM cell isolations from tibiae and femora yielded an equal number of cells in hyperglycemic and control C57BL/6J mice, whereas in FVB/N mice the hyperglycemic state was associated with a minor 1.3 fold increase in the number of BM-derived cells.

### Hyperglycemia Differentially Alters the Potential of BM Cells to Yield EPC, Mph, and DC

To determine the effects of hyperglycemia on the potential of BM progenitors to generate early EPC, total BM cells were harvested from hyperglycemic and control mice, and equal numbers of cells were cultured for 7 d to allow differentiation into EPC. We previously provided evidence that early outgrowth EPC, Mph, and DC may share a common myeloid progenitor pool in the BM (25). Therefore, the potential of the BM cells to differentiate into F480<sup>+</sup> Mph and CD11c<sup>+</sup> DC in M-CSF- and GM-CSF-stimulated cultures, respectively, was assessed in parallel to the EPC cultures. We observed on average a 40% reduction in the number of EPC, identified as cells positive for CD31 and acLDL (Figure 1A) in EPC cultures from BM of hyperglycemic mice from both strains. In contrast, the number of Mph was increased by approximately 50%. No differences were observed in the yield of DC in cultures

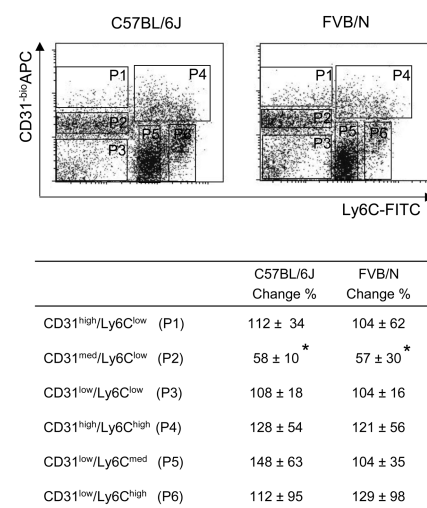


**Figure 1.** Hyperglycemia alters the differentiation potential of myeloid progenitors in BM. (A) Relative change in the number of bone marrow-derived EPC, Mph, and DC from STZ-treated mice (black bars) compared with control mice (white bars). Values for EPC, Mph, and DC derived from BM from mice treated with buffer are set to 100%. Top panel: number of CD31<sup>high</sup>/Dil-acLDL-positive, attaching cells in EPC cultures (C57BL/6J; 4 experiments,  $n_{\text{buffer}} = 18$  and  $n_{\text{STZ}} = 26$  and FVB/N; 5 experiments,  $n_{\text{buffer}} = 17$  and  $n_{\text{STZ}} = 23$ ). Middle panel: the number of F4/80-positive cells in M-CSF-stimulated Mph cultures (C57BL/6J; 3 experiments,  $n_{\text{buffer}} = 13$  and  $n_{\text{STZ}} = 18$  and FVB/N; 3 experiments,  $n_{\text{buffer}} = 13$  and  $n_{\text{STZ}} = 16$ ). Bottom panel: number of CD11c-positive cells in GM-CSF-stimulated dendritic cell cultures (C57BL/6J; 3 experiments,  $n_{\text{buffer}} = 13$  and  $n_{\text{STZ}} = 18$  and FVB/N; 3 experiments,  $n_{\text{buffer}} = 13$  and  $n_{\text{STZ}} = 16$ ). (B) Correlations between the number of cultured EPC (top panel), Mph (middle panel), and DC (bottom panel) and hyperglycemia as assessed by HbA<sub>1c</sub>. Representative experiment using BM from hyperglycemic C57BL/6J mice ( $n = 9$ ).

from both strains of mice. Figure 1B shows that the number of EPC obtained inversely correlated with peripheral blood HbA<sub>1c</sub> levels of individual mice, indicating that the observed decrease in EPC numbers directly relates to the degree of hyperglycemia. In contrast, the number of Mph showed a positive correlation with the degree of hyperglycemia, whereas the varying numbers of DC derived from the cultures showed no relationship with HbA<sub>1c</sub> levels. Taken together, hyperglycemia affects myeloid progenitor cells in BM and alters their potential to differentiate into different myeloid lineages.

### Hyperglycemia Has No Major Quantitative Impact on the Subpopulation Composition of BM Myeloid Progenitors

To evaluate whether the observed altered number of bone marrow-derived EPC and Mph from hyperglycemic mice was due to quantitative shifts in myeloid progenitor fractions of the BM, total BM was immunostained using ER-MP12 (anti-mouse CD31) and ER-MP20 (anti-mouse Ly-6C) and analyzed by flow cytometry. By using this combination of markers (26), we identified 6 distinct subpopulations of BM cells, each with varying degrees of lineage commitment



**Figure 2.** Hyperglycemia has no major quantitative impact on the myeloid progenitor fractions of the BM. BM of the control and hyperglycemic mice was harvested and immediately stained with anti-CD31 and anti-Ly-6C. Using this combination of markers, we identified 6 distinct populations (P1–P6). The populations consisted of: (P1) 70% blast cells and 25% lymphoid cells, (P2) lymphoid cells, (P3) erythroid cells, (P4) myeloid progenitors and plasmacytoid cells, (P5) granulocytes, (P6) 75% monocytes and 20% monocyte progenitors (26). For each subpopulation, the average increase or decrease in BM cell fractions from hyperglycemic mice is depicted as percentage compared with control mice. In the top panel representative scatter plots from hyperglycemic BM are shown for each strain. For both strains the average percentage and standard deviations were calculated from 3 experiments (C57BL/6J:  $n_{\text{buffer}} = 15$  and  $n_{\text{STZ}} = 18$  and FVB/N;  $n_{\text{buffer}} = 13$  and  $n_{\text{STZ}} = 14$ , \* $P < 0.001$ ).

and progenitor potential (Figure 2). As previously shown, Mph and DC can originate from CD31<sup>high</sup>/Ly6C<sup>low</sup> (P1), CD31<sup>+</sup>/Ly6C<sup>+</sup> (P4), and CD31<sup>low</sup>/Ly6C<sup>high</sup> (P6) fractions (26,27). However, short-term cultured EPC are derived mainly from the CD31<sup>+</sup>/Ly6C<sup>+</sup> fraction (25). When we compared the BM myeloid cell subpopulations from hyperglycemic and control mice, we found no significant quantitative differences in the three fractions from which EPC, Mph,

**Table 2.** Angiogenic and proinflammatory properties.<sup>a</sup>

	C57BL/6J Control (n)	C57BL/6J STZ (n)	P value	FVB/N Control (n)	FVB/N STZ (n)	P value
Angiogenesis length tubes, AU	0.47 ± 0.17 (8)	0.27 ± 0.12 (8)	0.03 <sup>b</sup>	0.77 ± 0.19 (12)	0.50 ± 0.20 (12)	0.02 <sup>b</sup>
Endocytosis EPC Δ geo mean	63.6 ± 18.0 (9)	78.8 ± 21.2 (15)	0.12	20.7 ± 17.9 (8)	42.3 ± 15.2 (8)	0.09
Endocytosis Mph Δ geo mean	47.0 ± 8.6 (3)	62.7 ± 8.0 (3)	0.13	78.3 ± 7.6 (3)	68.8 ± 11.4 (3)	0.28
Mixed lymphocyte reaction Δ 3H-thymidine incorporation (%)	100 (9)	176 ± 71 (15)	0.15	100 (6)	139 ± 27 (8)	0.08
IL-12p40 concentration, pg/mL	26.9 ± 13.2 (8)	64.4 ± 19.3 (8)	0.001 <sup>b</sup>	29.3 ± 13.5 (8)	56.6 ± 24.3 (8)	0.02 <sup>b</sup>
IL-12p70 concentration, pg/mL	78.4 ± 28.1 (8)	155 ± 52.5 (8)	0.006 <sup>b</sup>	62.7 ± 44.3 (8)	262 ± 63.1 (8)	< 0.001 <sup>b</sup>

<sup>a</sup>Functional analyses of EPC and Mph cultured for 7 d from control and hyperglycemic BM. Listed are the capacity of EPC-conditioned media to stimulate *in vitro* formation of tubular structures by HUVEC (n = 8, each group), the capacity of EPC and Mph to endocytose large dextran molecules labeled with FITC as measured by fluorescence-activated cell-sorting analyses and expressed as the delta geo mean (Δ), the capacity of EPC to stimulate T-cell proliferation as assessed by allogeneic mixed lymphocyte reaction (determined by measuring <sup>3</sup>H-thymidine incorporation and expressed in percentage compared with EPC from controls), and the expression of the production of proinflammatory cytokines IL-12p40 and IL-12 p70 by EPC after lipopolysaccharide stimulation (n = 8 per group).

<sup>b</sup>Statistically significant.

and DC can be derived in short-term cultures (Figure 2). The only subpopulation that was altered was the CD31<sup>med</sup>/Ly6C<sup>low</sup> lymphoid and HSC fraction (P2) that was 40% decreased in the BM of hyperglycemic mice of both strains. In line with the observation that the total cell number that can be harvested from the BM of these mice is unchanged or only marginally changed (Table 1), we conclude that hyperglycemia does not affect the quantitative composition of the myeloid progenitor subpopulations in the BM, but rather alters their differentiation potential.

### BM-Derived EPC from Hyperglycemic Mice Display a Proinflammatory Phenotype

To investigate whether the functional properties of EPC cultured from hyperglycemic BM are altered in comparison to those from control BM, we first determined the angiogenic potential of EPC-conditioned medium in an *in vitro* angiogenesis model. When HUVEC were seeded on matrigel and maintained for 14 h in conditioned medium of EPC derived from control C57BL/6J and FVB/N mice, tube formation was markedly stimulated when compared with nonconditioned medium (Table 2; nonconditioned media 0.37 ± 0.13 AU of tube formation, n = 3; P = 0.04 and P < 0.001, respectively). Conditioned media of EPC from

FVB/N mice induced more capillary formation than EPC-conditioned media of C57BL/6J mice (P = 0.01). In contrast, conditioned media of EPC from hyperglycemic mice supported tube formation by only 43% (C57BL/6J, P = 0.03) and 35% (FVB/N, P = 0.02) compared with the control values. Next we examined to what extent the different EPC displayed proinflammatory properties that are typical for myeloid antigen-presenting cells, for example, the ability to endocytose, the capacity to activate T-cell proliferation, and the expression of proinflammatory cytokines. Table 2 shows that EPC obtained from hyperglycemic mice displayed a trend toward a higher capacity to endocytose large dextran molecules, compared with EPC from normoglycemic mice. C57BL/6J EPC show a higher capacity to endocytose compared with FVB/N mice (P < 0.001). To assess whether the capacity of Mph derived from hyperglycemic mice to endocytose was altered, Mph were cultured from hyperglycemic and control mice. Mph derived from hyperglycemic BM from C57BL/6J mice showed a trend toward a higher capacity to take up large dextran molecules, whereas FVB/N-derived Mph showed a small but not significant decrease when derived from hyperglycemic BM.

When the EPC fractions were tested for their capacity to stimulate T-cell pro-

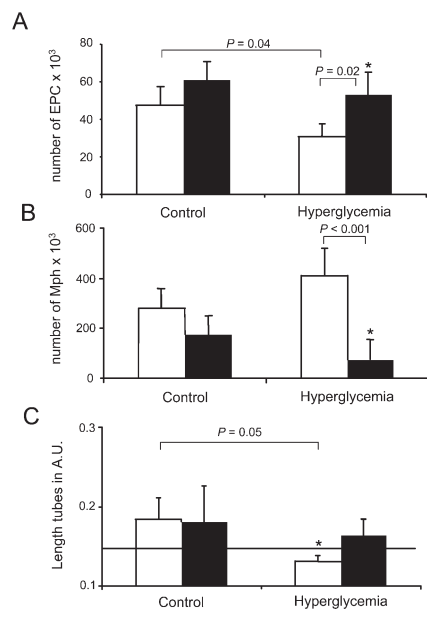
liferation in mixed lymphocyte reaction assays, again for both strains we observed a trend toward a higher inflammatory capacity of the EPC from hyperglycemic BM. This result was supported by our findings on the ability of the cells to produce IL-12 upon lipopolysaccharide stimulation. IL-12p70, the active form of the proinflammatory IL-12, can regulate T-cell-mediated immune responses by promoting Th1 development, and we have previously shown that it is the predominant IL-12 subtype produced by EPC(25). As shown in Table 2, IL-12p70 levels were elevated in the conditioned medium of EPC from hyperglycemic mice, ranging from 2.3-fold for C57BL/6J mice (P = 0.006) up to four-fold for FVB/N mice (P < 0.001). The IL-12p40 subunit was also about two-fold higher in supernatants from EPC derived from hyperglycemic mice compared with the supernatants of control EPC (C57BL/6J mice, P = 0.001; FVB/N mice, P = 0.02). Because elevated inflammatory properties were observed under hyperglycemic conditions, BM-derived EPC were further phenotypically characterized by flow cytometry analyses. However, when EPC from hyperglycemic and control mice were stained with either anti-F480 or anti-CD11c antibodies to reveal a more Mph- or DC-like phenotype, no significant differences could be observed (data not shown).

Taken together, these results indicate that EPC derived from hyperglycemic mice have a reduced angiogenic capacity but are more proinflammatory because they secrete more IL-12 and they show a trend for an elevated capability to endocytose exogenous material and to stimulate naïve T cells.

### Statin Treatment *in vitro* Restores Affected Progenitor Commitment and EPC Function of Hyperglycemic Mice

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) have been shown to be potent antiinflammatory agents (32). Moreover, they elevate EPC numbers *in vitro* (33) and *in vivo* (34) and interfere with the differentiation of monocytes toward Mph (35,36). Therefore, in separate experiments, we explored the effect of atorvastatin on hyperglycemia-induced effects on BM-derived EPC and Mph. BM cells of hyper- and normoglycemic C57BL/6J mice were cultured for 7 d to obtain EPC and Mph. As expected, lower numbers of EPC were derived from BM of hyperglycemic mice ( $P = 0.04$ ). When atorvastatin ( $0.1 \mu\text{mol/L}$ ) was present in EPC cultures from hyperglycemic mouse BM, the number of EPC were normalized to levels obtained from control mice (Figure 3A). Conversely, the number of Mph generated in M-CSF-stimulated cultures of both hyperglycemic and control BM was markedly reduced in the presence of atorvastatin ( $P < 0.001$ ; Figure 3B).

To evaluate the effect of atorvastatin on EPC function, an *in vitro* angiogenesis assay was performed with conditioned medium of EPC cultured in the presence of atorvastatin. As shown in Figure 3C, we again observed significant reduction in tube formation when HUVEC were subjected to conditioned media of EPC derived from hyperglycemic mice ( $P = 0.05$ ). This loss in paracrine angiogenic capacity was attenuated when the EPC from hyperglycemic mice were cultured in the presence of atorvastatin.



**Figure 3.** Atorvastatin reverses hyperglycemia-induced changes in progenitor commitment and EPC function. Numbers of EPC (A) and Mph (B) cultured from BM of control and hyperglycemic mice ( $n = 6$  per group). (C) Tube formation in arbitrary units (AU) in response to medium conditioned by EPC from control and hyperglycemic mice ( $n = 6$  per group) cultured in the absence (white bars) or presence of atorvastatin (black bars). The horizontal line represents tube length nonconditioned medium.

### DISCUSSION

Diabetes can be envisioned as a disease characterized by a chronic systemic inflammatory state disturbing the function of multiple vital body systems, in particular the vasculature (28,29). We hypothesized that hampered differentiation of EPC from myeloid progenitor cells in the BM might contribute significantly to the suboptimal endothelial repair under hyperglycemic conditions. Indeed, we observed a marked 40% reduction in the number of EPC that could be cultured from BM of mice that were made hyperglycemic by streptozotocin treatment, while at the same time the potential of BM to generate macrophages was increased by 50%. Similar to what we previously observed for the number of EPC that could be cultured from peripheral blood-mononuclear cells

from patients with type 1 diabetes (9), the observed reduction was inversely correlated with HbA<sub>1c</sub> levels, suggesting a causal role for hyperglycemia. In addition, EPC derived from hyperglycemic murine BM displayed impaired angiogenic activity. Again, EPC from patients with type 1 diabetes are likewise impaired in angiogenicity (9). Our findings confirm that EPC-mediated neovascularization may be impaired in diabetes due to a reduction in number and angiogenic capacity of the circulating EPC, caused by their defective development from myeloid BM progenitors. Indeed, others have shown that when progenitor cells derived from a hyperglycemic background are transplanted into mice they fail to augment ischemia-induced neovascularization (12,13) and have decreased capacity for re-endothelialization following arterial injury (37). However, our results also demonstrate that EPC derived from hyperglycemic BM display a proinflammatory phenotype, because their capacities to endocytose, to activate T-cells, and to produce IL-12 are increased. By mRNA expression profiling we recently demonstrated that circulating human EPC from patients with type 1 diabetes overexpress numerous proinflammatory genes known to be associated with hyperglycemia and oxidative stress, including osteopontin, plasminogen activator type 1, lectinlike oxidized LDL receptor, thrombomodulin, and type IV collagen (38). Together these data demonstrate that hyperglycemia may already, at the level of the BM, alter the differentiation and functional fate of progenitor cells. This may limit the use of BM cells in diabetic patients as a source for cell therapy to restore impaired vascular remodeling and repair. In this respect, it is of interest that *ex vivo* treatment with an HMGCoA reductase inhibitor can reverse the hyperglycemia-induced alterations in progenitor fate, suggesting that such a strategy can be used to improve cell therapy in the clinical setting of diabetes. Our data suggest that when EPC from a diabetic background arrive at sites of ischemia or vascular injury, the proinflammatory nature of these cells may contribute to an adverse (immune)

response that may be proatherogenic or contribute to the formation of neointima. EPC dysfunction was previously proposed to explain the proatherogenic nature of transplanted bone marrow–mononuclear cells from apolipoprotein E–knockout mice (39) and may limit the direct use of autologous progenitor cell transplantation for therapeutic angiogenesis in patients with ischemic vascular disease (40,41).

Surprisingly, the reduction in the number of EPC obtained from hyperglycemic BM was associated with a concomitant increase in the ability of these BM cells to generate Mph, whereas the number of DC that could be obtained with appropriate stimulation was not changed. We have previously shown that Mph and DC can be derived from common myeloid progenitor cell subsets in murine BM (26,27), and recent findings by Fogg *et al.* confirm this notion because they indicate that Mph and DC share a common BM progenitor (42). Here, we observed that the frequency of the different myeloid progenitor subsets in BM was not changed by hyperglycemia. Therefore, we propose that myeloid lineage differentiation potential of the BM progenitors was skewed as a consequence of the hyperglycemic state. Our observations suggest that inflammatory cytokines or elevated redox signaling may directly regulate the differentiation of various myeloid lineages from progenitor cells. Nuclear factor  $\kappa$ B is a major transcription factor responsive to cytokines and reactive oxidant species. Indeed, recent studies indicate that nuclear factor  $\kappa$ B, acting via IRF4, directly influences DC versus macrophage differentiation (43).

Recently we demonstrated that EPC, Mph, and DC show a significant phenotypic overlap and that a major source of EPC from the BM is the CD31<sup>+</sup>/Ly6C<sup>+</sup> fraction that predominantly contains myeloid progenitor cells that can also generate Mph and DC (25). This myeloid origin of EPC is consistent with the observation that CD34<sup>low</sup>CD14<sup>+</sup> cells in peripheral blood are a major source of human EPC (21). Assuming that the EPC,

the Mph, and the DC all originate from a common myeloid progenitor, we speculate that the proinflammatory milieu in diabetes not only skews the differentiation of the myeloid progenitor cell toward the Mph but that it does so at the cost of the generation of EPC. This scenario would imply that hyperglycemia-dependent alterations on cell-fate specification are not restricted to embryonic development (44) but may also affect the differentiation of adult progenitor cells. The ultimate proof of this concept may involve the use of techniques that monitor clonal expansion of the myeloid progenitor cells and HSC-like cells and are the subject of current investigations.

HMG-CoA reductase inhibitors (statins) have been shown to increase the number of circulating EPC in animal models (33,34) and in patients with stable coronary artery disease (45). Also, statins generate potent antiinflammatory actions (32) and can improve properties of dysfunctional EPC populations *in vitro* (4,34,45). In addition, statins appear to modulate the *in vitro* differentiation of monocytes to Mph (36). Therefore, we assessed whether the *in vitro* addition of atorvastatin reversed diabetes-associated changes in the EPC and Mph cultures. Indeed, we observed that atorvastatin restored EPC differentiation to control levels and also strongly decreased the elevated number of Mph generated in the BM cultures from hyperglycemic mice. Also, the angiogenic capacity of the EPC derived from hyperglycemic BM was partially recovered when cells were cultured in the presence of atorvastatin.

In conclusion, our study shows that EPC dysfunction in diabetes originates in the BM in association with hyperglycemia-induced alteration in differentiation of myeloid progenitor cells. The inflammatory nature of EPC in hyperglycemia may not only impair EPC function but may also contribute to premature atherosclerosis when these cells incorporate into the vessel walls. Our observation that statins can, at least in part, counteract these effects may not only provide a helpful tool to elucidate the molecular

mechanism underlying EPC dysfunction but may also contribute to the beneficial effect of statin therapy on the circulating levels of EPC in patients.

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## DISCLOSURE

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