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Immune Cells Mimic the Morphology of Endothelial Progenitor Colonies In Vitro

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ABSTRACT

Endothelial progenitor cells (EPC) are considered powerful biologic markers for vascular function and cardiovascular risk, predicting events and death from cardiovascular causes. Colony-forming units of endothelial progenitor cells (CFU-EC) are used to quantify EPC circulating in human peripheral blood. The mechanisms underlying colony formation and the nature of the contributing cells are not clear. We performed subtractive CFU-EC analyses to determine the impact of various blood cell types and kinetics of protein and gene expression during colony formation. We found that CFU-EC mainly comprise T cells and monocytes admixed with B cells and natural killer cells. The combination of purified T cells and monocytes formed CFU-EC structures. The lack of colonies after depletion or functional ablation of T cells or monocytes was contrasted with effective CFU-EC formation in the absence of CD34$^+$ cells. Microarray analyses revealed activation of immune function-related biological processes without changes in angiogenesis-related processes during colony formation. In concordance with a regenerative function, soluble factors derived from CFU-EC cultures supported vascular network formation in vitro. Recognizing CFU-EC formation as the result of a functional cross between T cells and monocytes shifts expectations of vascular regenerative medicine. Our data support the move from a view of circulating EPC toward models that include a role for immune cells in vascular regeneration. STEM CELLS *2007;25:1746–1752*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Regenerative stem cell therapy is a therapeutic concept based on the assumption that stem cells (SC) and progenitor cells bear the potential to support organ regeneration after ischemic, metabolic, or toxic injury. Mechanisms include vascular and somatic repair [1, 2]. Since endothelial cells (EC) can function as dynamic regulators of their surrounding somatic environment, particular attention has been paid to the role of EC and endothelial progenitor cells (EPC) after ischemic organ damage [3, 4]. An additional effect of bone marrow-derived mature cells of the immune system has been identified that may explain some of the variability in experimental models aiming to understand regenerative stem cell therapy [5–7]. Considerable controversy exists, however, regarding the nature and biological function of bone marrow-derived EPC, which are thought to play a central role during vascular regeneration [8, 9].

Over the past decade, different assays have been developed to study circulating potential endothelial progenitors [10]. The various published protocols can generally be divided into short-term (usually 4–10 days) differentiation of virtually nonproliferating cells as represented by (a) the colony-forming units of endothelial progenitor cells (CFU-EC) [11, 12] and (b) circulating angiogenic cell assay [11, 13], and late (typically after more than 10 days) outgrowth of highly proliferating blood outgrowth endothelial cells (BOEC) [14] or endothelial colony-forming cells (ECFC) [15]. CFU-EC are considered to provide an in vitro readout to enumerate circulating EPC [11]. The strong correlation between number of CFU-EC and vascular function led to the introduction of CFU-EC as a surrogate biologic marker for cardiovascular function and cumulative cardiovascular risk [12]. The initial data of Hill et al. [12] have been confirmed through other clinical studies with a standardized, commercially available assay. These studies show that the severity of coronary artery disease inversely correlates with the individual CFU-EC number [16, 17]. In one clinical trial, cumulative event-free survival significantly increased with increasing levels of CFU-EC. The significant association between decreased CFU-EC numbers and an increased risk of a major cardiac event even allowed the prediction of death from cardiovascular causes [17]. Complementary clinical results show that improved survival after acute lung injury is correlated with a higher CFU-EC count [18]. Despite the estimated enormous clinical relevance of data derived from this particular assay, the exact nature of progenitors considered to contribute to CFU-EC formation has not yet been elucidated. Delineation of candidate cells is mainly hampered by the fact that the majority of marker antigens does not allow a definitive distinction between hematopoietic and endothelial lineage [8].

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Taking into consideration the actual numbers of blood-borne cells attributable to an EC-associated phenotype may be one way to select candidate cells contributing to CFU-EC formation in vitro. More than 20 CFU-EC clusters have been obtained from 1 ml of peripheral blood of relatively healthy subjects [12]. Circulating $CD146^+$ EC (CEC) with high proliferative potential (also termed BOEC) have been measured in normal individuals at a frequency of 2.6 ± 1.6 cells per milliliter in whole blood [19]. Since the majority of CEC represent EC derived from vessel walls, they are excluded from CFU-EC analysis in the assay by an initial preplating step via fibronectin adherence [12, 14, 17]. The $CD34^+/KDR^+$ proposed hemangioblast, originally discovered as a hematopoietic SC, has been described as circulating at a frequency of 3–5 cells per milliliter in the blood of normal individuals [20, 21]. It is not known whether $CD34^+/KDR^+$ cells can adhere to fibronectin or whether they might contribute to CFU-EC formation. Another supposed EPC type has been observed in vitro at an extraordinarily high number of more than 2×10^4 nonproliferating acetylated low density $lipoprotein^+/lectin^+$ cells obtained from 1 ml of peripheral blood after only 4–6 days of angiogenic culture. It has been established, however, that these are actually monocytes that display an endothelial phenotype [8, 22–25]. In contrast, a rare bone marrow myeloid progenitor-derived cell type has recently been shown to contribute to vascular homeostasis in vivo in a parabiotic mouse model [26]. Since EPC are candidate cells for vascular regenerative therapy, it is of additional critical importance to delineate their origin to enable the targeted selection of regenerative cells. We therefore used a battery of cytomic, proteomic, and genomic analyses to elucidate the composition of CFU-EC.

Materials and Methods

CFU-EC Assay

Mononuclear cells (MNC) were obtained from heparinized peripheral blood of 19 healthy male volunteers (ages 19–62 years) according to institutional guidelines. Cell isolation and CFU-EC assays were performed as previously described using the EndoCult system (Stem Cell Technologies, Vancouver, BC, Canada, http://www.stemcell.com) [16, 17, 25, 27]. In brief, MNC were either mock-sorted or magnetically sorted for either $CD14^+$ (monocytes), $CD2^+$ (T cells), $CD34^+$ (hematopoietic progenitor cells, EC, and putative EPC), $CD19^+$ (B cells), or $CD56^+$ (natural killer [NK] cells; all from Miltenyi Biotec, Bergisch Gladbach, Germany, http://www.miltenyibiotec.com). Two rounds of column enrichment resulted in effective cell purification (>90%) or depletion (e.g., ≤0.01% remaining $CD34^+$ cells) of the respective cell type as confirmed by flow cytometry. The role of the presence or absence of the cells of interest in CFU-EC formation was evaluated by subtractive analyses. Mock-sorted, positive-sorted, depleted, and reunified cell fractions were cultured in fibronectin-coated wells at a seeding density of 5×10^5 cells per cm^2. After 48 hours, nonadherent cells were transferred into new coated wells, and colony formation per well (1 million cells per 2-cm^2 well) was counted at day 5. Potential CFU-EC inhibition by monoclonal antibodies against T cells, and tumor necrosis factor (TNF)-α was assessed using 1 μg/ml anti-CD3 (OKT3; Orthoclone; Ortho Biotech Inc., Raritan, NJ, http://www.orthobiotech.com) or 1 μg/ml anti-TNF-α (infliximab; Centocor, Leiden, The Netherlands, http://www.centocor.com), respectively, and compared with 1 μg/ml mouse IgG1 (MOPC21; Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com). Photographs were taken with a Coolpix 4500 digital camera (Nikon Instruments, Melville, NY, http://www.nikonusa.com) using a Diaphot 300 inverted microscope (Nikon).

Protein and Gene Expression Analyses

Immunohistochemical staining, flow cytometry, and real-time reverse transcription-polymerase chain reaction (RT-PCR) were performed [25, 28]. Immunohistochemistry of typical CFU-EC colonies was performed using mouse anti-human von Willebrand factor (vWF) (1.2 μg/ml; DakoCytomation, Glostrup, Denmark, http://www.dakocytomation.com), vimentin (2.4 μg/ml; DakoCytomation), and CD45 (0.5 μg/ml; Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com) antibodies compared with mouse IgG1 isotype control antibodies in appropriate concentrations. Human umbilical vein EC (HUVEC) served as positive controls for vWF staining. The peroxidase reaction was visualized with diaminobencidine (DakoCytomation). In flow cytometry experiments, freshly obtained or CFU-EC-derived cells at days 2 and 5 (adherent and nonadherent) were analyzed for surface expression of CD3, CD14, CD19, CD34, CD56, CD45 (antibodies obtained from BD Biosciences, San Diego, http://www.bdbiosciences.com), CD133 (Miltenyi), and vascular endothelial growth factor receptor 2 (VEGF-R2) (Sigma-Aldrich) on a FACSCalibur instrument (BD Biosciences). Multiplex human cytokine detection was used to measure the concentration of Eotaxin, granulocyte macrophage-colony-stimulating factor, interferon (IFN)-α, IFN-γ, interleukin (IL)-1β, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, inducible protein (IP)-10, IL-10, IL-12, IL-13, IL-15, IL-17, monocyte chemoattractant protein-1, monokine induced by interferon-gamma, macrophage inflammatory protein (MIP)-1α, MIP-1 β, regulated on activation normal T expressed and secreted, VEGF, and TNF-α from 50-μl aliquots of CFU-EC-derived cell culture supernatant following the manufacturer's instructions (Upstate, Waltham, MA, http://www.upstate.com).

The mRNA expression levels for the endothelial and hematopoietic cell-associated markers CD105, vascular endothelial growth factor receptors (VEGF-R1, 2, and 3), CD31, CD34, CD146, vWF, CD14, and CD45 of MNC before and after CFU-EC formation were determined using inventoried TaqMan gene expression assays in a quantitative real-time RT-PCR (RTQPCR) system (7900 HT; Applied Biosystems, Foster City, CA, http://www.appliedbiosystems.com). Single target PCR data are shown normalized to β-actin as the mean ± SEM log2-transformed expression ratio of CFU-EC (day 5) versus start MNC (day 0) of three independent experiments.

The ABI 1700 Expression Array System (Applied Biosystems) was used for full genome expression profiling. Two biological replicates (RNA derived from CFU-EC of two healthy donors compared with their respective start MNC tested on a total of four arrays) were analyzed. A starting amount of 2 μg of total RNA from each CFU-EC and MNC population was used for one round of linear amplification and labeling according to the manufacturer's instructions. The labeled probes were hybridized to Human Genome Survey Microarray, V2.0 (Applied Biosystems). Significantly upregulated genes in the course of CFU-EC culture were identified and annotated with respect to their involvement in biological processes and pathways using the Protein Analysis Through Evolutionary Relationships (PANTHER) database analysis software (SRI International, Menlo Park, CA, http://www.pantherdb.org). Results were stored in the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress; a MIAME checklist and all regulated genes are shown in supplemental online Table 1).

Vascular Network Formation

To obtain functional endothelial cells, umbilical cord blood (UCB)-derived EC were generated by culturing UCB-MNC ($1–3 \times 10^7$ cells per cm^2) in collagen-coated six-well plates in EGM-2 (Cambrex, Walkersville, MD, http://www.cambrex.com) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, http://www.hyclone.com) [15]. UCB-EC and human microvascular and umbilical vein EC (Cambrex) were used for comparative EC function analyses in Matrigel assays (Chemicon, Temecula, CA, http://www.chemicon.com) [25]. Test EC were used after passages 2–3 to study vascular network formation upon incubation of 8,000 EC per 96 wells with culture supernatants derived from effective (e.g., MNC-derived) versus noneffective CFU-EC assays (e.g., after depletion of monocytes or enrichment of $CD34^+$ cells). Network branches were photographically documented (Nikon), and photographs were com-

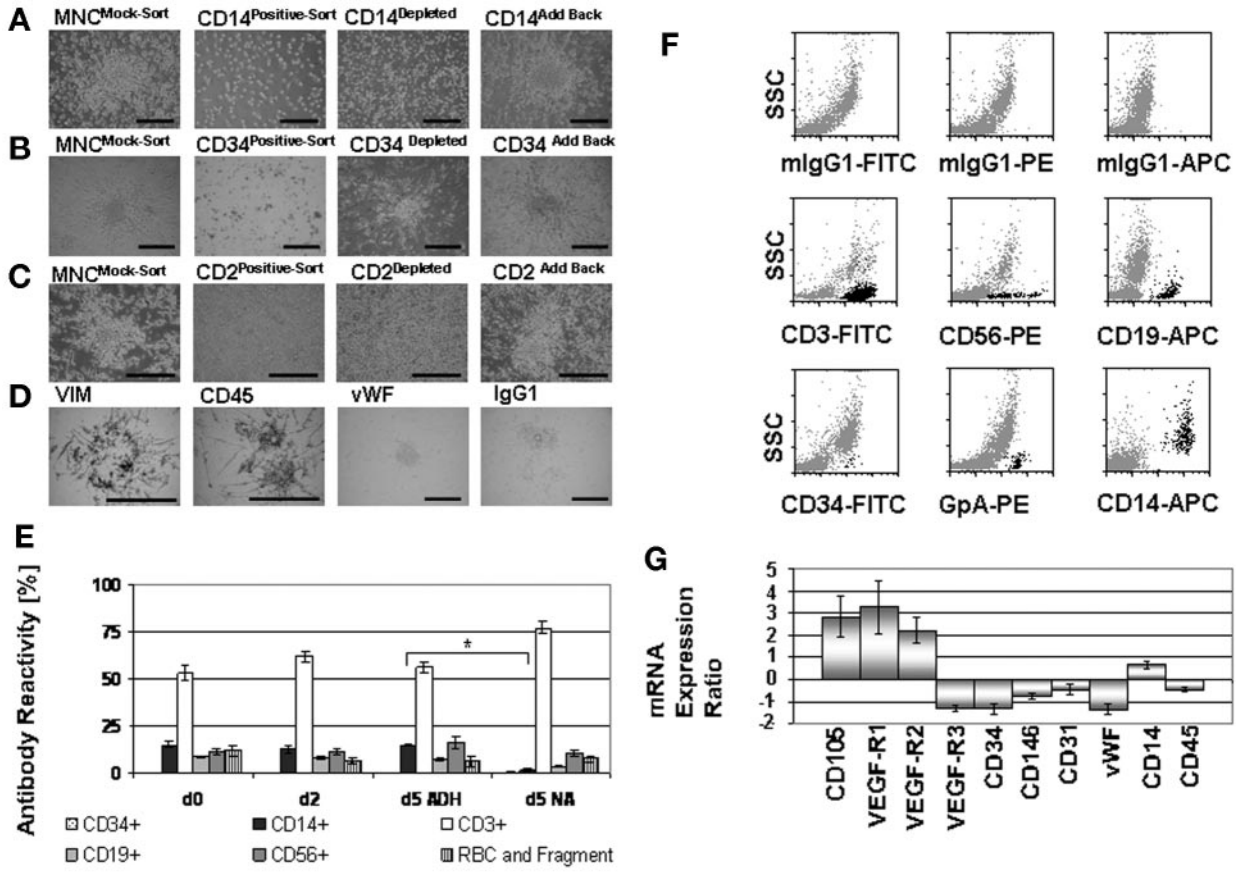

Figure 1. Colony-forming units of endothelial progenitor cells (CFU-EC) formation requires monocytes and T cells and does not depend on $CD34^+$ cells. Typical CFU-EC developed from mock-sorted MNC, not from $CD14^+$ and CD14-depleted cells, and reconstituted by adding back $CD14^+$ to CD14-depleted cells ($p = .294$; $n = 6$) **(A)**. $CD34^+$ cells did not form CFU-EC. Colony formation was unaffected in CD34-depleted ($p = .722$) and add-back cultures versus mock-sorted cells ($p = .053$; $n = 3$) **(B)**. CFU-EC development was abrogated by CD2 sorting or CD2 depletion and could be partially restored in CD2 add-back cultures versus mock-sorted cells ($p < .05$; $n = 3$) **(C)**. Immunohistochemistry showed VIM and CD45 but no vWF staining ($n = 3$) **(D)**. Scale bars = 200 μm. CFU-EC analyzed by flow cytometry. d5 NA cells were monocyte-depleted (*, $p = .018$; $n = 7$) **(E)**. Representative dot plots of a d5 ADH analysis including the appropriate isotype controls are depicted **(F)**. Log2 mRNA expression ratio of CFU-EC versus d0 MNC was determined by quantitative real-time reverse transcription-polymerase chain reaction. ($n = 3$) **(G)**. Abbreviations: ADH, adherent; APC, allophycocyanin; d, day; FITC, fluorescein isothiocyanate; GpA, glycophorin A; mIgG, mouse IgG; MNC, mononuclear cells; NA, nonadherent; PE, phycoerythrin; RBC, red blood cell; SSC, side scatter; VEGF-R, vascular endothelial growth factor receptor; VIM, vimentin; vWF, von Willebrand factor.

posed so that they displayed the entire wells (not shown). EC branches were counted by two independent investigators. Because of high inter-rater reliability (>0.93), mean values were able to be calculated. The numbers of branches after incubation with conditioned medium from effective CFU-EC were set to 100% and compared with branch counts derived from cultures with noneffective CFU-EC supernatants.

Statistical Analyses

Unless otherwise stated, data are shown as mean ± SEM. SPSS 14.0 software (SPSS, Inc., Chicago, http://www.spss.com) was used for statistical analyses. Statistical differences were assessed using the nonparametric paired Wilcoxon test, where applicable. Significance was set at $p < .05$.

RESULTS

Monocytes and T Cells Contribute to CFU-EC

In a first series of experiments, we confirmed our earlier observation that effective CFU-EC formation is strictly dependent on the presence of monocytes in the starting MNC preparation [25]. CFU-EC formation could be reconstituted by adding the sorted monocytes back to the monocyte-depleted cells, thus excluding an inhibitory function of the particular antibody (Fig. 1A). Since EC and various types of progenitors including EPC express the sialomucin CD34, we next performed a subtractive analysis of CFU-EC formation in the absence or presence of $CD34^+$ cells. Effective CD34 depletion as confirmed by flow cytometry did not alter CFU-EC formation (Figs. 1B, 2A). $CD34^+$ cells sorted to >90% purity were unable to form CFU-EC. Adding back the $CD34^+$ sorted cells did not influence CFU-EC formation (Fig. 1B). In contrast, depletion of T cells abrogated CFU-EC formation, as did the culture of purified T cells, whereas the mixture of $CD2^+$ sorted and CD2-depleted cells reconstituted colony formation. Although T cell- and monocyte-depleted preparations were enriched with $CD34^+$ cells, a total lack of CFU-EC formation was observed (Figs. 1C, 2B, 2C). Depletion of B cells and NK cells did not influence CFU-EC formation (Table 1). We next performed a detailed analysis by flow cytometry of the cellular composition over the time course of the colony assay. At all time points, more than 99% of the nucleated cells were $CD45^+$ hematopoietic cells. As estimated, starting MNC (day 0 [d0]) and the cells transferred at d2 mainly contained T lymphocytes together with sizeable fractions of monocytes, B cells,

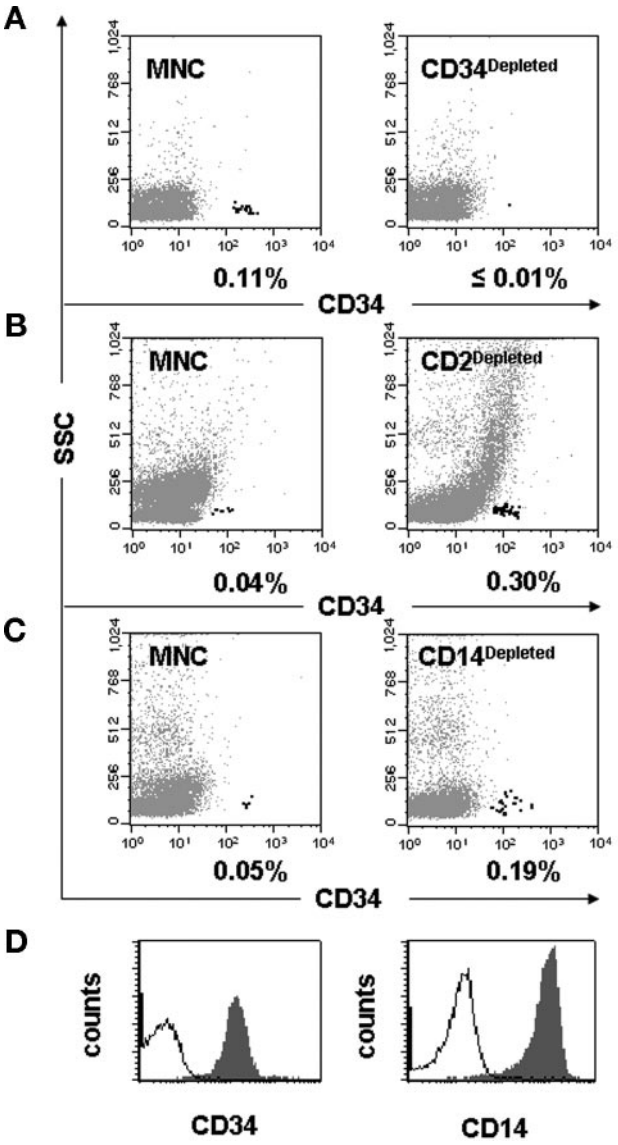

Figure 2. Efficacy of CD34 depletion and enrichment of $CD34^+$ cells by T cell and monocyte depletion prior to culture of colony-forming units of endothelial progenitor cells. Representative flow cytometry dot blots show that CD34-depletions were effective **(A)**. T cell or monocyte depletion resulted in CD34 enrichment within a mixture of either monocytes with $CD34^+$ **(B)** or T cells with $CD34^+$ **(C)** cells ($n \geq 3$ for all experiments). Sorted $CD34^+$ and $CD14^+$ cells (gray-filled histograms) and the respective isotype controls (open histograms) are depicted for comparison **(D)**. Abbreviations: MNC, mononuclear cells; SSC, side scatter.

and NK cells and a minute fraction of $CD34^+$ cells. The composition of the final adherent CFU-EC assay product (d5 adherent), however, did not differ significantly from that of the starting material (Fig. 1E, 1F). The nonadherent proportion (d5 nonadherent) of cells was significantly depleted of monocytes (Fig. 1E). $CD34^+/VEGF\text{-}R2^+$ cells were not detected, although more than 2 million events were analyzed. CD133 expression was ≤0.01% at any time point (data not shown). Immunohistochemical analysis of effective CFU-EC showed reactivity with the mesodermal marker vimentin and the hematopoietic cell-associated kinase CD45. We did not succeed in finding vWF reactivity in CFU-EC when using the antibodies in a concentration that resulted in a positive staining of positive control

Table 1. Subtractive CFU-EC experiments

Blood cell type subjected to CFU-EC analyses[a]	CFU-EC count per well	
	Number	%
MNC mock sort	29.4 ± 7	100 ± 25
MNC CD14-depleted	0	0
MNC $CD14^+$-selected	0	0
CD14 add-back	19.5 ± 5	66 ± 18
MNC mock sort	25.7 ± 10	100 ± 40
MNC CD34-depleted	24.4 ± 11	95 ± 43
MNC $CD34^+$-selected	0	0
CD34 add-back	15.6 ± 8	61 ± 31
MNC mock sort	28.4 ± 3	100 ± 10
MNC CD2-depleted	0	0
MNC $CD2^+$-selected	0	0
CD2 add-back	18.2 ± 3	64 ± 9
$CD2^+$ pure and $CD14^+$ pure	19.3 ± 10	68 ± 36

[a]The role of monocytes ($n = 6$), $CD34^+$ cells ($n = 3$), and T cells ($n = 3$) was studied in CFU-EC assays. Mean number of colonies in control MNC mock sorts were normalized to 100%. Depletion of $CD19^+$ B cells and $CD56^+$ natural killer cells did not abrogate CFU-EC formation, resulting in 129% and 104% of control cluster number in additional experiments. Mean ± SEM. Abbreviations: CFU-EC, colony-forming units of endothelial progenitor cells; MNC, mononuclear cells.

HUVEC and a negative staining of equally concentrated isotype control antibodies (Fig. 1D; data not shown). Comparative RTQPCR analysis of CFU-EC-derived versus day 0 MNC confirmed the immunohistochemistry data by showing a more than twofold downregulation of vWF, VEGF-R3, and CD34. A fourfold to eightfold upregulation of CD105, VEGF-R1, and VEGF-R2 was evident. No differential expression of CD146, CD31, CD14, and CD45 could be observed in the course of CFU-EC culture (Fig. 1G).

The most surprising result was that purified T cells together with purified monocytes alone were able to form the structures described as CFU-EC. An increase of consistently more than 10-fold in production of TNF-α was found in effective compared with monocyte-depleted and monocyte-enriched CFU-EC cultures. T-cell sorting resulted in a more than 50% lower TNF-α content in the respective noneffective (containing pure T

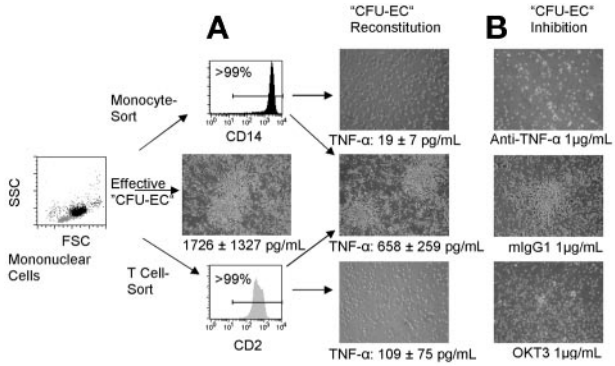

Figure 3. Monocytes plus T cells formed CFU-EC. Mononuclear cells formed typical CFU-EC. Purified monocytes or T cells did not originate CFU-EC, but CFU-EC reconstitution was achieved ($p = .128$) when these two populations were cultured together. Effective CFU-EC displayed high TNF-α levels in culture supernatants (mean ± SEM of three experiments) **(A)**. CFU-EC inhibition was induced by adding antibodies against TNF-α or T cells (OKT3) compared with control antibodies ($p = .406$; representative of three additional experiments) **(B)**. Abbreviations: CFU-EC, colony-forming units of endothelial progenitor cells; FSC, forward scatter; SSC, side scatter; TNF, tumor necrosis factor.

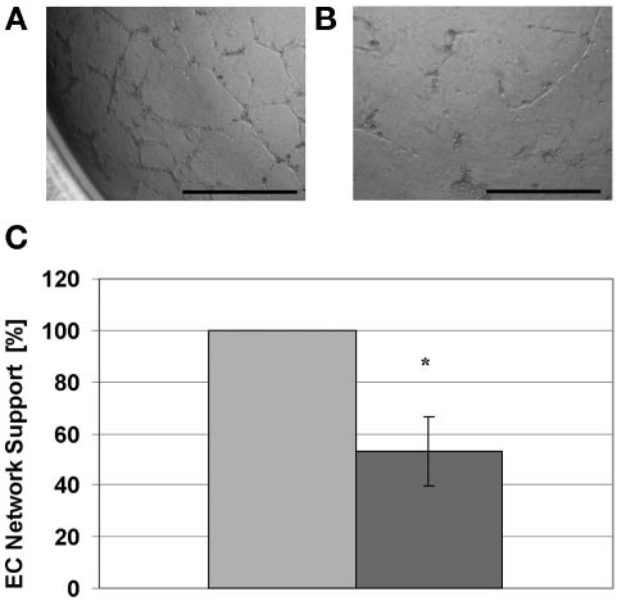

Figure 4. Vascular network formation is supported by immune cell-derived soluble factors. Representative human microvascular vein EC network formation in the Matrigel model system supported by supernatant derived from effective colony-forming units of endothelial progenitor cells (CFU-EC) **(A)** as compared to supernatant from noneffective CFU-EC **(B)**. Scale bars = 500 μm. Cumulative network branches of three types of test EC after incubation with effective CFU-EC-derived supernatant was set to 100% (▨) and compared with branch counts resulting from incubation with supernatant from noneffective CFU-EC (■) ($n = 5$; *, $p = .043$) **(C)**. Abbreviation: EC, endothelial cells.

cells or non-T cells) compared with effective CFU-EC (Fig. 3A; data not shown). Addition of antibodies against TNF-α resulted in a complete abrogation of CFU-EC development, as did the ablation of T-cell function achieved with the clinical anti-CD3 antibody OKT3 (Fig. 3B).

Vascular Network Formation Is Supported by Immune Cell Activation and Soluble Factor Production

Endothelial network formation provides a simple in vitro readout for vascular regenerative function. When seeded into extracellular matrix composites in the presence of an optimized proangiogenic culture medium, EC were able to form three-dimensional networks of vessel-like structures. Supernatants derived from effective CFU-EC cultures were able to replace the supportive function of the optimized EC medium (Fig. 4A). Network formation was significantly reduced in the presence of supernatants derived from noneffective CFU-EC (e.g., after depletion of monocytes or T cells; Fig. 4B, 4C). Multiplex cytokine display was performed to delineate the soluble factors potentially involved in the regulation of vascular regenerative function by immune cells. In addition to the above-described increased production of TNF-α in effective compared with monocyte-depleted CFU-EC cultures, we found that the concentrations of IP-10 and MIP-1β were reduced by more than 50% in noneffective CFU-EC cultures. Concentrations of other measured cytokines, including VEGF, showed minor variation that did not correlate with the effectiveness of CFU-EC (data not shown).

Whole transcriptome microarray analyses were used to obtain an overview of the biological processes underlying the formation of CFU-EC. Genes that were significantly upregulated ($p < .05$) in CFU-EC compared to their respective start MNC were grouped using the PANTHER database. The results strongly indicate an activation of mechanisms related to immunological processes and cell proliferation in the course of CFU-EC development. Processes including angiogenesis and vascular repair-associated extracellular matrix signaling were not affected (Table 2). A more detailed view revealed regulation of several genes involved in TNF signaling, in concordance with protein and functional data (supplemental online Table 1).

Table 2. Microarray genome analyses of CFU-EC

	p
Selected specifically upregulated biological processes[a]	
Immunity and defense	1.27×10^{-7}
Cytokine and chemokine-mediated signaling pathway	5.00×10^{-6}
Interferon-mediated immunity	1.10×10^{-5}
Cytokine/chemokine-mediated immunity	7.32×10^{-5}
Macrophage-mediated immunity	1.76×10^{-4}
Cell cycle	1.79×10^{-4}
T cell-mediated immunity	3.33×10^{-2}
MHC class II-mediated immunity	3.74×10^{-2}
Selected unaffected biological processes and pathways	
Angiogenesis	
Extracellular matrix protein-mediated signaling	
VEGF signaling pathway	
Endothelin signaling pathway	

[a]Supplemental online Table 1 shows all upregulated biological processes ($p < .05$) in CFU-EC-derived cells compared to start mononuclear cells and the list of genes identified for the selected categories including the respective fold change and p values.
Abbreviations: CFU-EC, colony-forming units of endothelial progenitor cells; MHC, major histocompatibility complex; VEGF, vascular endothelial growth factor.

DISCUSSION

We found that CFU-EC structures previously considered to display EPC progeny actually exhibit an immune cell aggregation in vitro. The data demonstrate that CFU-EC formation strictly depends on the presence of T cells and monocytes but not on $CD34^+$ progenitor cells. The fact that untouched $CD34^+$ cells are enriched in the CD14-depleted and CD2-depleted fractions further argues against a role of $CD34^+$ cells in the CFU-EC readout. Purified T cells, together with purified monocytes, were in fact able to form the CFU-EC clusters. The biological processes operative during this structural transit from dispersed MNC to monocyte-T cell clusters were restricted to immunity-associated pathways. The lack of direct angiogenic activity during CFU-EC development was supported by RTQPCR data that confirmed a stable hematopoietic $CD45^+$ phenotype without acquisition of the endothelial markers CD34, CD146, or vWF. Based on these data, we conclude that monocytes interact with T cells in this assay, mimicking the morphology of a structure so far considered to represent EPC progeny.

There are several possible explanations for the fact that purified T cells and monocytes in our add-back experiments did not restore CFU-EC plating efficiency to mean 100% of the control samples. Reconstitution to 68% ± 36% at least did not show a significant difference compared with control cultures ($p = .128$). As shown in Figure 3, TNF levels were lower in

CFU-EC reconstituted with pure T cells and monocytes. TNF has recently been found to influence CFU-EC derived from patients with active rheumatoid arthritis [29]. So far, we can only speculate whether the reduced TNF levels in vitro result from receptor (e.g., CD2 or CD14) engagement rather than representing a nonspecific consequence of cell manipulation. However, we could clearly show that engagement of CD3 or TNF depletion in vitro completely abrogated CFU-EC formation. Based on our results, a significant contribution of B cells, NK cells, and in particular $CD34^+$ cells to CFU-EC formation could be excluded.

During the review process of this paper, data were published by Yoder et al. [27] showing that CFU-EC are not EPC but are in fact myeloid cells that differentiate into phagocytic macrophages and fail to form perfused vessels in vivo. By using "naturally marked" hematopoietic cells from JAK2 1,849G>T polycythemia vera patients, Yoder et al. definitively found an elegant way not only to prove the hematopoietic lineage origin of the CFU-EC but also to demonstrate that CFU-EC are unable to form secondary EC colonies and fail to form perfused vessels in vivo. Following a detailed, extensive comparison to proliferating ECFC, these authors also concluded that the role of CFU-EC must be reexamined prior to further clinical trials [27].

Together with others' findings of a strong correlation of reduced CFU-EC counts with an increased risk of a cardiovascular event and death, our data may shed new light on the role of immune cells in cardiovascular homeostasis and regeneration [11]. Identifying the immune cell composition of the CFU-EC readout will be a step toward changing the interpretation of the results associated with that assay.

To date, the view on the role of the immune system in vascular homoeostasis has largely been restricted to a devastating function during the development of atherosclerosis. There is a large body of evidence that inflammation is an important risk factor for cardiovascular diseases. This particularly highlights the physiopathology of immune mechanisms that interact with metabolic risk factors to initiate, propagate, and activate lesions in the vascular system [30–37]. In contrast, vascular regeneration has been viewed as being solely dependent on sprouting angiogenesis and is currently considered to represent a complex interplay of somatic SC with a variety of circulating and, to some extent, bone marrow-derived progenitors [3, 4, 8]. In this scenario, the role played by EPC directly residing within the vessel wall is not fully understood [38, 39].

It is not yet clear whether the observed cytokine changes in the in vitro system correspond to those in vivo. TNF-α in particular has both potential beneficial effects, such as cardioprotection, and adverse effects, such as the development of atherosclerosis [40, 41]. It will be interesting to see whether the role of TNF-α in the CFU-EC in vitro system might indicate any influence of the now more frequently used anti-TNF antibody therapy on vascular homeostasis in vivo. There is a correlation between reduced numbers of the immune cell-derived colonies, previously termed CFU-EC, and cardiovascular events and death from cardiovascular causes. Further work is needed to establish whether this corresponds to treatable immune deviations that affect vascular homeostasis.

There is evidence that bone marrow-derived cells play a promotional role in in vivo vascular regeneration [5, 11, 42–45]. VEGF produced locally at the site of organ damage has been shown to be sufficient to initiate homing of bone marrow-derived myeloid admixed with nonmyeloid cells distinct from EPC. Retention of these immune cells in close proximity to sprouting vessels has been found to be mediated by a stromal-derived factor [5]. These results extend our understanding of a mostly evolutionary link between hematopoiesis and angiogenesis that is well recognized during embryonic development. Emerging concepts consider vascular regeneration as a complex interplay between somatic progenitors and stem cells interacting with supporting angio-competent bone marrow-derived myeloid cells [5, 43–46].

CONCLUSION

Considerable controversy exists regarding the nature of bone marrow-derived EPC and their role in vascular homeostasis and repair. Using a series of cytomic, proteomic, and genomic analyses, we found that the structures described as CFU-EC actually function as a cross between T cells and monocytes. Our observation that T cells can interact with monocytes to mimic CFU-EC parallels that of Yoder et al. [27], who demonstrated the hematopoietic origin of the CFU-EC structures through in vitro and in vivo experiments. Both types of studies established that CFU-EC are not EPC and thus support emerging models that separate the role of bone marrow-derived immune cells from that of somatic progenitors and stem cells [5, 26, 45]. From a clinical point of view, we need better insight into the complex interplay of resident and immigrating cell populations to select candidate cells for potential regenerative therapies. Highly proliferating endothelial progenitors, which can form vessels in vivo, represent one candidate cell fraction [19, 27, 38, 39]. Animal and patient data showing proangiogenic properties of various types of bone marrow-derived MNC and putative EPC will hopefully enable us to develop promising therapeutic regimens.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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