Endothelial Progenitor Cells and a Stromal Cell-derived Factor-1 α Analogue Synergistically Improve Survival in Sepsis

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Abstract

Rationale: Endothelial progenitor cells (EPCs) have been associated with human sepsis but their role is incompletely understood. Stromal cell-derived factor (SDF)-1 α facilitates EPC recruitment and is elevated in murine sepsis models. Previous studies have demonstrated that the SDF-1 α analog CTCE-0214 (CTCE) is beneficial in polymicrobial sepsis induced by cecal ligation and puncture (CLP) in mice.

Objectives: We hypothesized that exogenously administered EPCs are also beneficial in CLP sepsis and that CTCE provides synergistic benefit.

Methods: Mice were subjected to CLP and administered EPCs at varying doses, CTCE, or a combination of the two. Mouse survival, plasma miRNA expression, IL-10 production, and lung vascular leakage were determined. The *in vitro* effect of CTCE on miRNA expression and EPC function were determined.

Measurements and Main Results: Survival was improved with EPC therapy at a threshold of 10⁶ cells. In coculture studies, EPCs augmented LPS-induced macrophage IL-10 production. *In vivo* EPC administration in sepsis increased plasma IL-10, suppressed lung vascular leakage, attenuated liver and kidney injury, and augmented miR-126 and -125b expression, which regulate endothelial cell function and/or inflammation. When subthreshold numbers of EPCs were coadministered with CTCE in CLP mice they synergistically improved survival. We demonstrated that CTCE recruits endogenous EPCs in septic mice. In *in vitro* analysis, CTCE enhanced EPC proliferation, angiogenesis, and prosurvival signaling while inhibiting EPC senescence. These cellular effects were, in part, explained by the effect of CTCE on miR-126, -125b, -34a, and -155 expression in EPCs.

Conclusions: EPCs and CTCE represent important potential therapeutic strategies in sepsis.

Keywords: endothelial progenitor cell; sepsis; CTCE; microRNA

Septic shock with multiple organ failure is the leading cause of death in intensive care units and remains a major health problem in the United States. Disruption of the vascular barrier is a critical step in the development of multiple organ failure in sepsis (1–3). Endothelial progenitor cells (EPCs) are circulating endothelial precursor

cells associated with sepsis (4–7). A recent study demonstrated that circulating EPCs were increased in patients with sepsis, whereas nonsurvivors had significantly lower numbers of EPCs than survivors (8). Another study found an inverse relationship between the number of EPC cfu and organ dysfunction in sepsis (9).

EPC function is also altered in sepsis. Patschan and coworkers (10) demonstrated a reduced proliferative capacity of EPCs from patients with sepsis, whereas Luo and coworkers (11) showed alterations in EPC adhesion, migration, and angiogenesis capacities in a porcine hemorrhage-endotoxemia model. These findings,

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At a Glance Commentary

Scientific Knowledge on the

Subject: Sepsis leading to septic shock and multiple organ failure is the leading cause of death in intensive care units and remains a major health problem in the United States. The loss of vascular barrier integrity is an important step in the development of multiple organ failure in sepsis. To date, there is no effective treatment for the vascular barrier disruption of sepsis.

What This Study Adds to the

Field: We demonstrated that endothelial progenitor cells and the stromal cell–derived factor (SDF)-1 α analogue, CTCE, synergistically improve survival in murine polymicrobial sepsis. Endothelial progenitor cells and CTCE regulate microRNA expression and suppress lung vascular leakage. These studies may lead to novel therapeutic strategies to treat sepsis.

combined with observations that EPCs influence endothelial regeneration and repair (12, 13), serve as compelling reasons to further study the role of EPCs in sepsis.

Stromal cell–derived factor (SDF)-1 α is a critical mediator for ischemia-specific recruitment of circulating EPCs (14, 15). In our previous work, plasma SDF-1 α and circulating EPCs were increased in mice subjected to moderate cecal ligation and puncture (CLP) but not in mice subjected to severe CLP (16). This led to an exploration of the effects of the SDF-1 α analog CTCE in sepsis. Administration of CTCE suppressed plasma tumor necrosis factor (TNF)- α levels in LPS-induced shock and zymosan peritonitis and improved murine survival in severe CLP sepsis (16). In addition, other investigators demonstrated that CTCE enhanced survival of cord blood–derived CD34⁺ cells in mice (17). Thus, CTCE and EPCs may act in concert to reduce inflammation and promote endothelial integrity prompting us to further investigate their function in mouse CLP-induced sepsis.

MicroRNAs (miRNAs) are 21–23 nucleotide noncoding RNAs that regulate gene expression at the post-transcriptional

level. MiRNAs play a critical role in the regulation of inflammation and angiogenesis (18, 19). MiR-126 is the most abundant miRNA expressed during endothelial cell differentiation and genomic knockout of miR-126 increases vascular permeability (18–21). Conversely, miR-34a inhibits EPC angiogenesis and promotes inflammation-induced EPC senescence by suppression of silent information regulator 1 (Sirt1) (22). Furthermore, miR-155 and miR-125b are both expressed by endothelial cells (23, 24) and have been shown to modulate inflammation in sepsis models through targeting regulators of Toll-like receptor (TLR) signaling and TNF- α expression (25, 26). We hypothesized that EPC survival and function in sepsis would be modulated by CTCE by expression of miRNAs.

The current study investigated the effect of exogenous EPCs derived from human cord blood on murine CLP sepsis. We also examined if CTCE improves EPC function and provides a synergistic benefit in sepsis. The data demonstrate that CTCE and EPCs synergistically improve survival in CLP-induced severe sepsis. CTCE enhances EPC function and EPC prosurvival signaling regulated, in part, through specific miRNA expression. Some of our data have been previously reported in an abstract (27).

Methods

Human EPC Isolation, Culture, and Stimulation

Human EPCs isolated from cord blood were cultured as previously described (28). Cord blood samples were collected from umbilical veins during normal full-term, vaginal deliveries. Informed consent was obtained from the mother for all cord blood collections. The study was approved by the Institutional Review Board for Human Research at the Medical University of South Carolina.

For signaling protein expression, EPCs were serum starved for 6 hours and stimulated with CTCE (1 μ g/ml; Chemokine Therapeutics, Vancouver, BC, Canada) (16) for different time intervals. Phospho ERK 1/2, AKT, GSK3 β , and endothelial nitric oxide synthase (eNOS; Cell Signaling, Danvers, MA) were determined by Western blot. For miRNA expression, EPCs were treated with LPS (100 ng/ml) for 6 and 24 hours or CTCE

(1 μ g/ml) for 1, 6, and 24 hours or combined LPS (100 ng/ml) and CTCE (1 μ g/ml) for 24 hours. MiRNA-126, -34a, -155, and -125b were determined by real-time polymerase chain reaction (PCR).

CLP

CLP was performed in CD-1 mice (male, aged 7–8 wk) as previously described (29). Investigations conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee.

Mice were subjected to CLP and administered intravenous EPCs (1×10^6 cells) or human umbilical vein endothelial cells (HUVEC; 1×10^6 control cells) at 24 hours after CLP. To test the synergistic effect of EPCs and CTCE, the concentration of CTCE was decreased to a subprotective dose (1 mg/kg), whereas the number of EPCs was reduced to 1×10^5 cells. CTCE and EPCs were either administered alone or together. Mice received the antibiotic imipenem (25 mg/kg, subcutaneously) at 6, 24, and 48 hours after CLP. Survival was monitored for 7 days.

Circulating EPCs were quantified after mice were subjected to CLP and treated with or without CTCE (10 mg/kg) at 2 and 6 hours post-CLP. Mice received imipenem (25 mg/kg subcutaneously) at 6 hours post-CLP. Mice were sacrificed at 12, 18, and 24 hours post-CLP for assessment of EPCs in blood using flow cytometry.

Another group of mice was subjected to sham or CLP and injected with EPCs (1×10^6 cells, intravenously) at 6 hours, CTCE (10 mg/kg) at 2 and 6 hours, or EPCs (1×10^5 cells) together with CTCE (1 mg/kg) at 6 hours post-CLP. Serum or plasma and lung tissue were collected at 24 hours post-CLP. Plasma TNF- α , IL-6, and IL-10 levels were assayed by ELISA. Plasma aspartate aminotransferase, alanine aminotransferase activity (MUSC laboratory services), and creatinine (QuantiChrom creatinine assay; BioAssay Systems, Hayward, CA) were determined. Exosomes were isolated from serum using ExoQuick Exosome Precipitation Solution (System Biosciences, Mountain View, CA). MiRNA-126, -34a, -155, and -125b expression in serum exosomes were determined by real-time PCR. Lung permeability was determined using Evans blue dye as previous described (30). Lung tissues were fixed in 10% formalin

and sections were stained with hematoxylin and eosin.

Flow Cytometry Analysis of Mouse EPCs *In Vivo*

EPCs in peripheral blood were determined as described (10). Cells were stained with the following antibodies: FITC-antimouse-CD34 antibody (eBioscience, San Diego, CA) and PE-antimouse-FLK-1 (BD Biosciences, San Diego, CA).

Coculture of EPCs with Bone Marrow-derived Macrophage

Bone marrow was isolated from femurs of mice and bone marrow-derived macrophage (BMDM) cultured as described (29). BMDMs (1×10^6 cells per well) were seeded in 24-well plates. EPCs (2×10^5 cells per well) were added on the top of BMDM. Cells were pretreated with different concentrations of CTCE for 24 hours and stimulated with LPS 10 ng/ml in the presence of CTCE for 24 hours. LPS-induced IL-10 was determined by mouse IL-10 ELISA (eBioscience).

EPC Function Assay

EPCs (5×10^3 cells) seeded in 96-well plates were incubated with CTCE (1 ng/ml to 1 μ g/ml) for 24 hours. EPCs were incubated with MTT (Thiazolyl Blue, Tetrazolium Bromide, 0.5 mg/ml; Sigma, St. Louis, MO) for 4 hours and the reaction was stopped with solvent (100 μ l, 0.04–0.1 N HCl in isopropanol). Absorbance of converted dye was measured at 570 nm with background subtraction at 690 nm.

EPCs were treated with TNF- α (10 ng/ml) with or without SDF-1 α (10 ng/ml) or CTCE (1 μ g/ml) for 5 days. Culture medium was replaced with fresh medium with TNF- α , SDF-1 α , or CTCE daily. EPC senescence was determined by β -Galactosidase Staining Kit (Cell Signaling) (31).

EPCs (2×10^4 cells per well) were seeded in a 96-well plate coated with Matrigel Basement Membrane Matrix (BD Biosciences) and treated with CTCE (10 ng to 10 μ g/ml) or SDF-1 α (10 ng to 100 ng/ml) for 18 hours. Cells were stained with Calcein AM (8 μ g/ml; BD Biosciences) and visualized by fluorescence microscopy to assess tube formation.

Nitric Oxide Analysis

EPCs (4×10^5 cells per well) were seeded in 12-well plates and treated with CTCE (1 μ g/ml) in serum-free medium for 16 hours.

Cells were loaded with DAF-FM diacetate (Invitrogen, Carlsbad, CA) for 1 hour and detached with trypsin. Cells were fixed with 2% PFA and analyzed by flow cytometry.

miRNA Analysis and Transfection

Isolated microRNAs were amplified with miRNeasy and miScript SYBR Green PCR Kits (Qiagen, Valencia, CA). Hs RNU6-2 miRNA was used as internal control. EPCs were transfected with a miR-34a mimic, control siRNA, miR-126 inhibitor, or control inhibitor with HiPerFect transfection reagent (Qiagen).

Statistical Analysis

Data are expressed as the mean $\pm$ SEM. Statistical significance was determined by analysis of variance with Fisher probable

least-squares difference test or Student *t* test using GraphPad Prism software (La Jolla, CA). A *P* < 0.05 value was considered statistically significant.

Results

CTCE and EPCs Synergistically Improve Survival

Septic mice were administered EPCs (1×10^6 cells), control HUVECs (1×10^6 cells), or vehicle at 24 hours post-CLP. EPCs significantly improved survival at 7 days compared with vehicle or HUVEC control (87% vs. 33%; *P* < 0.05) (Figure 1A), whereas HUVECs (47%) did not significantly alter survival. Previous studies demonstrated that CTCE at a concentration of 10 mg/kg is beneficial in CLP-induced

Figure 1. CTCE and endothelial progenitor cells (EPCs) synergistically improve survival in cecal ligation and puncture (CLP)-induced sepsis. CD-1 mice (15 per group) were subjected to CLP or sham operation and (A) treated with EPCs or control human umbilical vein endothelial cells (HUVEC) or vehicle or (B) combination of subthreshold doses of CTCE and EPCs. Survival of mice was monitored for 7 days. Mice surviving in each group were labeled as survived mice/total mice. PBS = phosphate-buffered saline.

Figure 2. Effect of CTCE on cecal ligation and puncture (CLP)-induced endothelial progenitor cells mobilization. Murine CLP was induced in CD-1 mice treated with imipenem. CTCE (10 mg/kg) or vehicle was administered by subcutaneous injection at 2 and 6 hours after CLP. Percentage of blood CD34⁺/VEGFR2⁺ endothelial progenitor cells was determined by flow cytometry. **P* < 0.05 compared with sham groups, #*P* < 0.05 compared with CLP groups; n = 3–6 mice per group.

sepsis (16). Survival benefit of CTCE or EPCs alone was lost when CTCE was reduced to 1 mg/kg or the number of EPCs reduced to 1×10^5 cells. However, when these subthreshold doses of CTCE and EPC treatment were combined, survival from sepsis was synergistically improved compared with vehicle control (80% vs. 33%; *P* < 0.05) (Figure 1B).

CTCE Induces Endogenous EPC Recruitment

CLP sepsis with antibiotic treatment was induced in CD-1 mice. CTCE (10 mg/kg) or vehicle was administered by subcutaneous injection at 2 and 6 hours after CLP. CD34⁺/VEGFR2⁺ EPCs were determined in blood at 12, 18, and 24 hours post-CLP. CLP-induced sepsis resulted in decreased circulating EPCs 24 hours post-CLP. CTCE augmented recruitment of endogenous EPCs to peripheral blood during sepsis (Figure 2).

EPCs Increase LPS-stimulated IL-10 Production in BMDMs

LPS increased BMDM IL-10 expression, whereas EPCs treated with LPS did not up-regulate IL-10 expression (data not shown). To determine the effect of EPCs on macrophage function, EPCs were cocultured with BMDMs. EPCs significantly potentiated LPS-induced IL-10 production by BMDM (2.0 ± 0.1 fold vs. BMDM LPS

group) (Figure 3A). CTCE did not boost the effect of EPCs on macrophage function despite dose up-titration.

EPCs Increase Plasma IL-10, miR-126, and miR-125b and Decrease Vascular Leakage in CLP-induced Sepsis

Mice were subjected to sham or CLP and injected with EPCs or vehicle at 6 hours post-CLP. EPCs or subthreshold EPC and CTCE combination significantly increased plasma IL-10 production (3.3 ± 0.5 fold and 3.5 ± 0.6 fold, respectively) (Figure 3B) but had no effect on plasma TNF- α or IL-6 production (data not shown). EPCs decreased lung vascular leakage as shown by the Evans blue assay ($55 \pm 2\%$ decrease) (Figure 3C) and reduced inflammatory cell infiltration in the lung (Figure 3D). Furthermore, EPCs significantly reduced plasma aspartate aminotransferase and creatinine levels (Figure 3E). EPCs increased serum exosome miR-126 and -125b (3.1 ± 0.8 and 3.4 ± 0.4 fold, respectively) (Figure 3F) but had no effect on miR-34a and -155 expression (data not shown).

SDF-1 α and CTCE Alter EPC Function *In Vitro*

EPCs were treated with CTCE at escalating doses for 24 hours and proliferation was measured with MTT. CTCE at concentrations greater than or equal to 10 ng/ml significantly increased the proliferation of EPCs (1.21 ± 0.02 fold) (Figure 4A).

EPCs were also treated with TNF- α (10 ng/ml) with or without SDF-1 α (10 ng/ml) or CTCE (1 μ g/ml) for 5 days. TNF- α increased senescence in EPCs (3.6 ± 0.3 fold). Both SDF-1 α and CTCE decreased TNF- α -induced senescence ($59 \pm 3\%$ and $48 \pm 3\%$, respectively) (Figure 4B).

Finally, EPCs were treated with CTCE (1 μ g/ml) for 16 hours. NO levels were determined by DAF-FM diacetate. CTCE significantly increased NO production in EPCs (1.3 ± 0.1 fold) (Figure 4C).

EPCs were cultured in Matrigel matrix with or without SDF-1 α or CTCE for 18 hours and tube formation was measured. CTCE and SDF-1 α significantly increased total length of tubes and number of branch points per surface area. SDF-1 α at 100 ng/ml improved tube formation by 5.1 ± 0.6 fold. CTCE at 10^2 to 10^4 ng/ml also improved tube formation approximately

fivefold (Figure 4D). In aggregate, these data suggest that CTCE increases EPC proliferation, prevents TNF- α -induced senescence, and improves angiogenic function of EPCs.

CTCE Reverses Sepsis-induced Alterations of miRNA Expression

EPCs were treated either with CTCE (1 μ g/ml), LPS (100 ng/ml), or a combination of both for different time intervals and miRNA expression was determined. LPS decreased miR-126 and -125b expression, whereas CTCE increased miR-126 and -125b expression and reversed LPS-induced decreases (Figures 5A and 5B). LPS and CTCE both decreased miR-34a expression *in vitro*. Interestingly, CTCE also blocked LPS-induced decreases of miR-34a in spite of decreasing it on its own (Figures 5A and 5B). LPS increased miR-155 levels, whereas CTCE decreased miR-155 and reversed LPS-induced increases (Figures 5A and 5B).

Mice were subjected to CLP and treated with CTCE (10 mg/kg) at 2 and 6 hours post-CLP. MiRNAs were determined in exosomes isolated from mouse serum 24 hours post-CLP. MiR-126 and -125b were decreased by CLP and CTCE prevented this reduction (Figure 5C). Serum exosome miR-34a from CLP mice was not different from control animals. However, CTCE significantly decreased exosomal miR-34a (Figure 5C). CLP induced increases of miR-155, whereas CTCE significantly abrogated this increase (Figure 5C). These data demonstrate that CTCE reverses LPS and CLP-induced miRNA expression change *in vitro* and *in vivo*.

CTCE Increases ERK 1/2 Signaling and EPC Proliferation via miR-126 and Increases AKT/GSK3 β /eNOS Signaling

EPCs were treated with CTCE (1 μ g/ml) for different time intervals. ERK 1/2, AKT, GSK3 β , eNOS, and Spred-1 signaling were determined by Western blot. CTCE time-dependently increased ERK 1/2 (Figure 6A) and AKT (Figure 6B) phosphorylation, which peaked at 30 minutes. GSK3 β and eNOS were activated by CTCE at 30–90 minutes (Figures 6C and 6D). CTCE significantly decreased Spred-1 expression (Figure 6E). EPCs were transfected with a miR-126 inhibitor or control and treated with CTCE. In miR-126 inhibitor

Figure 3. Effects of endothelial progenitor cells (EPCs) on macrophage function and cecal ligation and puncture (CLP)-induced sepsis. (A) EPCs were cocultured with bone marrow–derived macrophage (BMDM), pretreated with CTCE (10 ng/ml to 1 μg/ml) for 24 hours, and stimulated with LPS (10 ng/ml) for 24 hours. IL-10 production was determined by ELISA. **P* < 0.05 compared with basal group, #*P* < 0.05 compared with BMDM stimulated with LPS group. *n* = 4 independent experiments. Mice were subjected to sham or CLP. EPCs or EPCs together with CTCE were injected intravenously at 6 hours post-CLP. Plasma IL-10 (B), Evans blue leakage (C), lung tissue damage (D), plasma aspartate aminotransferase (AST), alanine aminotransferase (ALT), and creatinine levels (E) and serum exosome miR-126 and -125b (F) were determined. Arrows indicate reduced alveolar air space and infiltration of inflammatory cells. **P* < 0.05 compared with sham group, #*P* < 0.05 compared with CLP group. *n* = 3–10 mice per group.

transfected cells, CTCE-induced ERK 1/2 activation and EPC proliferation was blocked (Figures 6F and 6G). Thus, CTCE-induced miR-126 expression may increase EPC proliferation and ERK 1/2 signaling by decreasing the expression of the miR-126 target Spred-1.

CTCE Reduces TNF-α-induced Senescence via Reduced miR-34a Expression

Sirt1 reduces endothelial cell senescence and is a known target of miR-34a (22). Expression of Sirt1 was determined in EPCs

treated with CTCE (1 μg/ml) for 30 minutes to 24 hours. CTCE significantly increased Sirt1 expression, which lasted for at least for 24 hours (Figure 7A). When a miR-34a mimic was transfected into EPCs, Sirt1 expression was decreased confirming that miR-34a targets and reduces Sirt1 expression (Figure 7B). EPCs were also transfected with control siRNA or miR-34a mimic and treated with TNF-α (10 ng/ml) with or without CTCE (1 μg/ml) for 5 days. TNF-α increased senescence, which was inhibited by CTCE in control siRNA transfected cells. In the

miR-34a mimic transfected cells, however, CTCE failed to prevent TNF-α-induced senescence (Figure 7C). Thus, CTCE may prevent TNF-α-induced senescence through suppression of miR-34a expression.

Discussion

Our findings demonstrated that exogenously administered human cord blood–derived EPCs are protective when delivered after the onset of CLP-induced

Figure 4. Effects of CTCE on endothelial progenitor cells (EPCs) function *in vitro*. (A) EPCs were stimulated with CTCE (1 ng/ml to 1 μ g/ml) for 24 hours. CTCE-induced EPC proliferation was determined by MTT assay. * $P < 0.05$ compared with basal group. $n = 5$ independent experiments. (B) EPCs were treated with tumor necrosis factor (TNF)- α (10 ng/ml) with or without stromal cell-derived factor (SDF)-1 α (10 ng/ml) or CTCE (1 μ g/ml) for 5 days. EPC senescence was determined by β -galactosidase staining Kit. * $P < 0.05$ compared with control group, # $P < 0.05$ compared with TNF- α group. $n = 6$ independent experiments. (C) EPCs were treated with CTCE (1 μ g/ml) for 16 hours. Nitric oxide levels were determined by DAF-FM diacetate and analyzed by flow cytometry. * $P < 0.05$ compared with control. $n = 3$ independent experiments. (D) EPCs were cultured in Matrigel matrix with or without SDF-1 α or CTCE at different concentrations for 18 hours. Cells were stained with Calcein AM and visualized by fluorescence microscopy. Representative pictures of tube-like structures and statistical analysis of total length of tubes and the number of branch points per field are shown. * $P < 0.05$ compared with basal group. $n = 4$ independent experiments.

sepsis. Moreover, subthreshold protective numbers of EPCs in combination with subthreshold protective doses of CTCE synergistically improved survival. Administration of EPCs to septic mice augmented plasma IL-10, miR-126, and miR-125b expression while suppressing lung vascular leakage and organ dysfunction. *In vitro* coculture studies revealed that EPCs augmented LPS-induced IL-10 production by BMDM. Also, CTCE

improved EPC function *in vitro* as evidenced by the prevention of TNF- α -induced EPC senescence and enhancement of EPC prosurvival signaling, proliferation, and angiogenesis in part through modulation of miRNA expression.

Several investigators have demonstrated that EPCs facilitate endothelial regeneration and repair in ischemic injury (32, 33). Recent studies have also shown that circulating EPCs are

associated with human sepsis (9, 10). In two studies, patients with sepsis were found to have increased numbers of circulating EPCs by flow cytometry compared with control subjects (10, 34). Alternatively, Cribbs and coworkers (9) reported that patients with sepsis have significantly reduced numbers of circulating EPCs. These conflicting findings likely stem from the latter group's use of cfu assay for EPC quantification, which can be biased by alterations in cell

Figure 5. Effect of CTCE on miRNA expression *in vitro* in endothelial progenitor cells and *in vivo* in cecal ligation and puncture (CLP)-induced sepsis. Endothelial progenitor cells were treated *in vitro* with CTCE (1 µg/ml, A) or LPS (100 ng/ml, B) with or without CTCE for different time intervals. CD-1 mice were subjected to CLP and treated *in vivo* with CTCE (10 mg/kg) at 2 and 6 hours post-CLP (C). Exosomes were isolated from mouse serum 24 hours post-CLP. MiRNA-126, -34a, -155, and -125b expression were determined by real-time polymerase chain reaction. * $P < 0.05$ compared with basal group, # $P < 0.05$ compared with 24-hour LPS stimulation groups or CLP groups. $n = 4$ independent experiments.

proliferation. Indeed, Patschan and coworkers (10) simultaneously reported increased circulating EPCs in sepsis by flow cytometry and reduced EPC proliferative capacity using the CFU assay. Importantly, EPC number and function also are associated with survival in sepsis. Sepsis survivors had significantly more circulating EPCs than nonsurvivors, whereas Cribbs and coworkers (9) found that lower CFUs were associated with more organ dysfunction in sepsis. Our current studies are consistent with the existing literature by demonstrating that mice subjected to the severe sepsis experience reductions in circulating EPC number at 24 hours. However, beyond potential prognostic value of peripheral EPCs, the therapeutic value of increasing EPC number, via

exogenous administration, to improve survival in CLP-induced sepsis was demonstrated.

The protective effect of EPCs demonstrated in this study is consistent with previous work demonstrating that administering mesenchymal stromal cells is beneficial in CLP-induced sepsis (35). Activated mesenchymal stromal cells reprogram macrophages by releasing prostaglandin E_2 , which in turn activates macrophage EP2 and EP4 receptors resulting in reduced TNF- α and IL-6 production but increased IL-10 production (35). In our study, when BMDMs were cocultured with EPCs and stimulated with LPS, they similarly produced higher IL-10 levels compared with BMDMs stimulated with LPS alone.

Accordingly, some of the beneficial effects of progenitor cells, such as mesenchymal stromal cells or EPCs in sepsis, may be through the ability of the donor cells to reprogram macrophages toward a lesser inflammatory phenotype (M2 macrophage polarization) (36). Indeed, in another study, progenitor cells derived from human embryonic stem cells were shown to interact with CD11b⁺ cells and ameliorate sepsis-induced lung injury (37). The ability of cocultured human embryonic stem cells to reduce the inflammatory phenotype of CD11b⁺ cells was attributed to NO production possibly derived from eNOS (37). We found that CTCE significantly increased EPC AKT/GSK3 β /eNOS signaling and NO production.

Figure 6. Effects of CTCE on signaling protein expression in endothelial progenitor cells (EPCs). EPCs were treated with CTCE (1 $\mu\text{g/ml}$) for different time intervals. (A) Phospho-ERK 1/2, (B) Phospho-AKT, (C) Phospho-GSK3 β , (D) Phospho-eNOS, and (E) Spred-1 were determined by Western blot. (F and G) EPCs were transfected with control or miR-126 inhibitor and treated with CTCE (1 $\mu\text{g/ml}$). ERK 1/2 activation and EPC proliferation were determined. The statistical analysis was performed on densitometry scans. * $P < 0.05$ compared with basal group, # $P < 0.05$ compared with CTCE group. $n = 3$ –5 independent experiments.

In addition to altering the inflammatory phenotype, we have demonstrated that EPCs improve lung vascular integrity in sepsis while simultaneously altering serum levels of miR-126 and -125b, miRNA known to influence

endothelial cell function. Interestingly, we also detected exosomes produced by EPCs containing miRNA, including miR-126 (data not shown). MiR-126 is a critical regulator of endothelial cell function and barrier integrity and could represent

a pathway through which EPCs exert their protective effects on vasculature in sepsis. It is unlikely that EPCs improve vascular barrier integrity solely by mechanical intercalation into areas of injured endothelium because of their rapid effect on

Figure 7. Effect of CTCE and miR-34a on Sirt1 expression and tumor necrosis factor (TNF)- α -induced senescence. (A) Endothelial progenitor cells (EPCs) were treated with CTCE (1 μ g/ml) for different time intervals or (B) transfected with miR-34a mimic. Sirt1 expression was determined by Western blot. α -tubulin was used as loading control. The statistical analysis was performed on densitometry scans. $n = 3$ independent experiments. * $P < 0.05$ compared with basal group. (C) EPCs were transfected with control siRNA or miR-34a mimic and treated with TNF- α (10 ng/ml) with and without CTCE (1 μ g/ml) for 5 days. Senescence of EPC was determined by β -galactosidase staining kit. * $P < 0.05$ compared with control siRNA transfected control group; # $P < 0.05$ compared with TNF- α group. \$ $P < 0.05$ compared with miR-34a mimic transfected control group. $n = 4$ independent experiments.

vascular integrity and the xenogeneic nature of the current study. We hypothesize instead that human EPCs hone to injured endothelium and restore vascular barrier integrity through modulation of inflammatory and antiinflammatory cytokines and through a paracrine release of exosomal miRNA, which regulate endothelial cell-cell junctions. These effects may persist after immune-mediated clearance of xenogeneic EPCs. This hypothesis is beyond the scope of the present study and is currently under investigation.

Our results also suggest that CTCE modulates EPC miRNA expression while simultaneously altering EPC function; specifically, sepsis reduces serum levels of miR-126 and that CTCE reverses this decline. Similarly, CTCE induced miR-126 expression in EPCs, whereas LPS has the converse effect. We further demonstrated that CTCE decreased the expression of the

miR-126 target *spred-1* known to inhibit cell proliferation through inhibition of Ras-mediated ERK 1/2 activation (38, 39). Accordingly, we showed that CTCE increased EPC proliferation and ERK 1/2 activation, which was abolished when EPCs were transfected with miR-126 inhibitors. In aggregate, these data suggest that CTCE enhances EPC proliferation via up-regulation of miR-126 and subsequent ERK 1/2 activation.

CTCE also reduced senescence of EPCs. In sepsis, proinflammatory cytokines (e.g., TNF- α) accelerate endothelial cell or EPC senescence-like dysfunction (9–11). MiR-34a has been shown to target the longevity gene Sirt1, which regulates senescence in EPCs (22). We found that CTCE significantly decreases miR-34a expression and increases Sirt1 expression in EPCs. This mechanism may, in part, explain our observation that CTCE reduces TNF- α -induced senescence of EPCs.

Finally, CTCE also reversed the effect of LPS and sepsis on other EPC miRNA expression. LPS increased miR-155 and decreased miR-125b expression in EPCs, whereas CTCE had the opposite effect. Identical patterns were seen *in vivo* in the serum of septic mice treated with CTCE. MiR-155 and -125b are implicated in the regulation of inflammatory response in sepsis (25, 26). MiR-155 targets SHIP1 and SOCS1, which are negative regulators of TLR signaling and target Bcl 6, which inhibits TNF- α gene transcription. Similarly, miR-125b inhibits TNF- α expression (40). The effect of sepsis-related alterations in miR-155 and -125b expression, therefore, would be enhanced TLR signaling and TNF- α expression perhaps leading to an overexuberant inflammatory response. These data suggest that CTCE may counter these effects of sepsis on EPC miRNA expression and, thereby, attenuate proinflammatory response.

There are limitations of the current study. We have shown that EPCs reduce hepatic and renal dysfunction in sepsis that is an indirect measure of microcirculatory failure. In addition, EPC improved lung vascular leak and injury. Collectively, these results raise the possibility that EPCs improve vascular barrier integrity in experimental sepsis. However, we have not fully explored the effect of EPCs on microvascular circulation with capillary microscopy. Additionally, although we have demonstrated that a therapeutic threshold exists for the amount of EPCs delivered, we have not yet characterized the time-to-treatment threshold for this therapy. A better understanding of when EPC treatment is required and a deeper exploration of the protective mechanisms of EPCs in sepsis may facilitate future novel treatment strategies for human sepsis.

In summary, EPCs improve survival and reduce organ failure in experimental sepsis while modulating the inflammatory response and enhancing vascular barrier integrity. CTCE provides synergistic benefit by enhancing EPC function in part by altered miRNA expression. Collectively, these studies demonstrate that EPCs with or without CTCE have therapeutic potential and may constitute a novel treatment strategy for sepsis. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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