Hydrogel-embedded endothelial progenitor cells evade LPS and mitigate endotoxemia

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Ghaly T, Rahadi MM, Weber M, Rahadi SM, Bank M, Grom JM, Fallon JT, Goligorsky MS, Ratliff BB. Hydrogel-embedded endothelial progenitor cells evade LPS and mitigate endotoxemia. Am J Physiol Renal Physiol 301: F802–F812, 2011. First published July 20, 2011; doi:10.1152/ajprenal.00124.2011.—Sepsis and its complications are associated with poor clinical outcomes. The circulatory system is a well-known target of lipopolysaccharide (LPS). Recently, several clinical studies documented mobilization of endothelial progenitor cells (EPCs) during endotoxemia, with the probability of patients’ survival correlating with the rise in circulating EPCs. This fact combined with endotoxemia-induced vascular injury led us to hypothesize that the developing functional EPC incompetence could impede vascular repair and that adoptive transfer of EPCs could improve hemodynamics in endotoxemia. We used LPS injection to model endotoxemia. EPCs isolated from endotoxic mice exhibited impaired clonogenic potential and LPS exerted Toll-like receptor 4-mediated cytotoxic effects toward EPCs, which was mitigated by embedding them in hyaluronic acid (HA) hydrogels. Therefore, intact EPCs were either delivered intravenously or embedded within protecin-coated HA hydrogels. Adoptive transfer of EPCs in LPS-injected mice improved control of blood pressure and reduced hepatocellular and renal dysfunction. Specifically, EPC treatment was associated with the restoration of renal microcirculation and improved renal function. EPC therapy was most efficient when cells were delivered embedded in HA hydrogel. These findings establish major therapeutic benefits of adoptive transfer of EPCs, especially when embedded in HA hydrogels, in mice with LPS-induced endotoxemia, and they argue that hemodynamic and renal abnormalities of endotoxemia are in significant part due to developing incompetence of endogenous EPCs.

lipopolysaccharide; stem cell therapy; microcirculation; renal function; sepsis

Sepsis occurs in 751,000 Americans and accounts for 215,000 deaths annually (2). Sepsis-related acute kidney injury (AKI) is associated with a 70–80% mortality rate. During sepsis, the immune response “storm” leads to widespread damage of parenchymal and endothelial cells, deformation, and apoptosis causing endothelial shedding (1). While systemic hypotension accompanies the immune system response, locally there is severe renal vasoconstriction leading to AKI.

Lipopolysaccharide (LPS), a component of the outer membrane of gram-negative bacteria, is a critical mediator of endotoxemia, multiple organ dysfunction syndrome, and endotoxic shock. Vascular endothelium is a well-established target of LPS. The response is not uniform and there are regional and species-specific differences in the intensity of responses to LPS (1). Proinflammatory and procoagulant changes, increased vascular permeability, and impaired vasomotion are common manifestations. Structural companions of these aberrations include endothelial cell swelling, vacuolization, and denudation (36).

Renal vasculature is among the most sensitive vascular beds to LPS challenge, thus contributing to AKI. LPS-induced fibrin deposition has been consistently reported in the kidney where it is confined to glomerular and peritubular capillaries in different species (10, 41, 42). Apoptotic cell death has been demonstrated in bovine glomerular endothelial cells subjected to LPS (22). In contrast to systemic actions of LPS leading to profound hemodynamic perturbations, when regional renal microvasculature is affected, as in a model of thrombotic microangiopathy, reparative processes are initiated earlier after insult (14). One possible explanation for this difference may lie in the degree to which endothelial progenitor cells (EPCs) are damaged by the disease process and the extent to which this damage impedes their engagement in vascular/organ regeneration.

The dynamics of EPCs in septic patients have been the focus of several investigations, which demonstrated abnormalities in the level of circulating EPCs that correlated with the outcome (7, 24, 35). The possibility that LPS affects EPC functions has ignited interest in therapeutic possibilities of adoptive transfer of EPC. Specifically, we transplanted EPC embedded in hyaluronic acid hydrogels (HA hydrogels) and released on demand by dissolving hydrogels with hyaluronidase, a strategy we found to be cytoprotective against other systemic insults (28).

MATERIALS AND METHODS

Sepsis models. The animal study protocol was in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee. Two models of sepsis were utilized in experiments, LPS-induced endotoxemia and cecal ligation and puncture (CLP). FVB/NJ male mice aged >16 wk and weighing 25–27 g were used for all experiments. For LPS-induced endotoxemia, a single intraperitoneal injection of 3.5 mg/kg LPS (from Escherichia coli Serotype 0127:B8, Sigma, St. Louis, MO) injection was applied. For CLP-induced sepsis, through a midline incision in anesthetized mice, cecal ligation was performed distal to the ileocecal valve, and a 21-gauge needle puncture of the ligated cecum was made. Eighteen hours after either LPS injection or CLP, samples were taken for subsequent analyses.

Colony-forming unit assay. EPCs were obtained from mice 18 h after injection of LPS or vehicle (normal saline). Briefly, bone marrow was harvested by flushing femurs with DMEM, homogenized, and mononuclear cells were separated by Ficoll-paque density gradient centrifugation. The endothelial colony-forming cell (ECFC) assay, current standard in identifying EPC from bone marrow, peripheral blood, and umbilical cord blood (13, 15, 45), was used to detect and quantify bone marrow EPC. In brief, mononuclear cells obtained after

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Ficoll gradient centrifugation were plated (5 x 10^6 cells) on fibronectin-coated dishes and resuspended in EPC medium (EGM-2 supplemented with Stem Cell Stimulatory Supplement, STEMCELL Technologies, Vancouver, Canada). Early outgrowth colonies (4–7 days) are of hematopoietic phenotype and have a short life span. Long-term culture colonies (>50 cells), or late outgrowth colonies, formed, as expected, between 7 and 21 days and are considered to be EPCs. These late-outgrowth cells are clonally distinct from the early-outgrowth cells and are considered to represent true EPC as they express primary endothelium surface antigens such as von Willibrand factor, CD31, CD105, CD146, KDR, CD144, while also expressing the stem cell marker CD34. In contrast to the early-outgrowth cells, the late-outgrowth cells do not express myeloid or hematopoietic antigens, such as CD14, CD45, or CD115. Late-outgrowth cells, when replated continue to form colonies, display robust proliferative potential and have angiogenic ability showing neovascularization properties when administered in vivo.

Cell culture. A previously established line of mouse embryonic EPC was used for all adoptive transfer experiments (12). These were isolated mesodermal cells from murine embryos at embryonic day 7.5. The isolated embryonic cells displayed stem cell-like growth potential and a stable phenotype in culture (12). RNA analysis revealed these embryonic cells express endothelial-specific genes tie-2, thrombomodulin, and von Willibrand factor as well as the early mesodermal marker fgf-3 (12). EPCs were cultured in dishes coated with 0.1% gelatin (Sigma), in DMEM high glucose (Invitrogen, Carlsbad, CA) with 20% FBS, 1% penicillin/streptomycin, and 1% nonessential amino acids (Invitrogen). Cells were labeled using CellTracker CM-DiD according to the manufacturer’s instructions (Invitrogen).

HA hydrogel formation and degradation. HA hydrogels [composed of thiol-modified denatured collagen, thiol-modified HA, and the crosslinker polyethylene glycol diacrylate (PEGDA)] were prepared using the HyStem Hydrogel Kit (Glycosan Biosystems, Salt Lake City, UT) as previously described (28). Additional components, including 50 μg/ml pronectin (Sigma) and EPCs, were added before solidification with crosslinker. Pronectin was added to HA hydrogels because previous studies indicated better viability of stem cells in hydrogels coated with this polymeric RGD (arginine-glycine-aspartic acid peptide domain) (28). For cell release, gels were digested using 300 U/ml collagenase and 100 U/ml hyaluronidase (Sigma).

LPS diffusion rate in HA hydrogels. HA hydrogels were prepared using PEGDA crosslinker concentrations of 2, 4, and 8%. EPCs were embedded in gels and gels were plated in EPC medium. LPS fluorescently labeled with Alexa Fluor 594 (Invitrogen) was added to medium at a concentration of 5 μg/ml. HA hydrogels were monitored with fluorescence microscopy to determine the rate and depth of penetration of the fluorescently labeled LPS. In addition, staining of embedded EPCs with a Live/Dead assay (Invitrogen) allowed for analysis of gel depth at which penetrating LPS adversely affected the viability of embedded cells.

Live/dead assay. To assess the effects of LPS on EPCs in and out of HA hydrogels, the LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen) was used in parallel with fluorescence microscopy to evaluate the viability of cells. LPS was added to the hydrogels at various concentrations, and the viability of EPCs was assessed using the Live/Dead assay. The results showed a dose-dependent decrease in cell viability with increasing LPS concentration.

Fig. 1. Lipopolysaccharide (LPS) diffusion into hydrogels fabricated with varying crosslinker concentrations. A, C, E: depth of LPS penetration as measured by incubating hydrogels of varying crosslinking concentration (2, 4, and 8%) with fluorescently tagged LPS for 90 min (T values represent time). Images (B, D, F) of corresponding hydrogels with embedded endothelial progenitor cells (EPCs) incubated with LPS for 24 h before live/dead cell assay. In 2% hydrogels (E, F), LPS diffuses rapidly and deeply into the hydrogel causing massive amounts of cell death as evidenced by the lack of live cells at the periphery of the hydrogel and the presence of dead cells closer to the center. Higher crosslinked hydrogels including 4 and 8% (C, D) demonstrated decreased LPS penetration and decreased diffusion rate. Insets depict ×4 zoomed corresponding images; n = 5. Scale bar: 100 μm.
TLR4 receptor on their surface membrane. Cells were trypsinized and stained with 7AAD and TLR4 antibodies (Abcam) for FACS (flow cytometry) analysis. Data were acquired using a FACSscan cytometer equipped with a 488-nm argon laser and a 620-nm red diode laser and analyzed using CellQuest software (Becton Dickinson, San Jose, CA). The setup of FACS was performed using unstained and secondary antibody-stained cells.

**In vivo HA hydrogel implantation.** Two sites were used for in vivo implantation of EPCs embedded in HA hydrogels, subcutaneously in the ear and subcapsularly in the kidney. The ear was chosen for implantation because it allows for easy subsequent digestive enzyme injection directly in the hydrogel to dissolve the implant and mobilize EPC. For ear implantation, mice were anesthetized with ketamine (60 mg/kg) and xylazine (6.6 mg/kg) and 10 μl of HA hydrogel loaded with fluorescently tagged (CellTracker CM-DiI) EPC (5 × 10^6 cells each side or 38 × 10^6 per kg of body wt) were injected in both ears. Immediately after LPS injection, ear implants were injected with collagenase (300 U/ml) and hyaluronidase (100 U/ml) to permit mobilization of embedded EPCs. For kidney implantation, mice were anesthetized as above, kidneys were exposed by a midline incision, and 10 μl of HA hydrogel loaded with fluorescently labeled (CellTracker CM-DiI) EPCs (5 × 10^6 cells each side or 38 × 10^6 per kg of body wt) were injected under the renal capsule of both kidneys. Kidney gel implants were not subsequently digested with collagenase/hyaluronidase enzymes due to their endogenous production by kidneys (8, 16, 18, 19, 32). After recovery from gel implantation procedures, LPS was injected intraperitoneally (3.5 mg/kg) or CLP was performed to induce sepsis. EPCs were also delivered by tail vein injection immediately after sepsis induction (1 × 10^6 cells).

**Liver function assay.** Liver damage was quantified by alanine transaminase (ALT) and aspartate transaminase (AST) colorimetric assay (Cayman Chemical, Ann Arbor, MI). Briefly, mice were given LPS and treated with either intravenous EPCs or HA hydrogel (with and without EPCs). After 18 h, mice were killed, plasma was collected, and the assay’s manufacturer’s protocol was applied.

**Laser Doppler flowmetry of renal blood flow and analysis of renal function.** Renal blood flow was evaluated by laser Doppler flowmetry (LDF) 18 h after sepsis induction and delivery of EPCs by either hydrogel implantation or intravenous delivery. Septic mice were sedated with xylazine/ketamine, a midline incision was made to access kidneys, and blood flow was measured with laser Doppler flow probes. Blood flow within the cortical and medullary regions was measured. To assess renal function in septic mice, mice were killed 18 h after sepsis induction and blood was obtained by cardiac puncture. Serum creatinine concentration was measured using a creatinine assay kit (Abcam, Cambridge, MA).

Blood pressure measurement was conducted using a noninvasive blood pressure monitoring system and accompanying software NIBPM v1.23 (Columbus Instruments).

**Chemokine/cytokine effects.** Plasma was evaluated for chemokine/cytokine levels 18 h after mice were injected with LPS intraperitone-
ally and treated with EPCs either intravenously or via HA hydrogel implantation subcutaneously into the ear. Chemokine/cytokine levels were analyzed using the multiplex LumineX 100 system (Luminex, Austin, TX) and an anti-mouse chemokine/cytokine panel I assay (Millipore, Billerica, MA), according to the manufacturer’s protocol.

**Morphologic studies.** For acute studies, 18 h after LPS injection and adoptive transfer of EPC by intravenous delivery or hydrogel, mice were killed and kidneys and livers were fixed in 4% paraformaldehyde and paraffin embedded. After being sectioned (5-μm section thickness), kidney and liver samples were stained with hematoxylin and eosin and morphology was analyzed.

For long-term effects, two mo after LPS injection and adoptive transfer of EPC by IV delivery or hydrogel, mice were killed, kidneys fixed in 4% paraformaldehyde, followed by 30% sucrose, cryosectioned at 10 μm and stained with Masson’s trichrome. Area of fibrosis was quantified using Image J Software (NIH).

**Statistical analysis.** Data are presented as means ± SE. For multiple comparisons between groups, a one-way ANOVA with Tukey’s posttest was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA). Differences were considered significant at \( P < 0.05 \).

**RESULTS**

We used the ECFC assay to detect abnormalities in EPC colony formation in endotoxemia. EPCs were obtained from mice injected with 3.5 mg/kg LPS or a vehicle (normal saline). LPS resulted in reduced clonogenic ability (13 ± 2.3 colonies per 10^6 cells in control and 1 ± 0.5 in LPS). The data suggest that endotoxemia affects EPC competence, thus potentially contributing to the observed hemodynamic abnormalities (7). Finding of impaired EPC competence in endotoxemia also justified attempts at implementing such cell therapy that would allow physical protection from LPS. Based on our previous findings that embedding of EPCs in bioartificial scaffolds...
afforded protection against adriamycin cytotoxicity (28), we explored the possibility that a similar strategy may be efficient in protecting EPCs from LPS. Moreover, we previously demonstrated that coating of scaffolds with pronectin significantly improved cell preservation (28), hence the same protocol was used in studies described below.

First, we examined whether the embedding of EPC into HA hydrogel or any of its components, including denatured collagen, thiol-modified HA, and the crosslinker PEGDA, at the concentrations used to form HA hydrogels, affected the viability or the phenotype of EPC. HA hydrogels did not affect embedded EPC competence, morphology, viability (28), or phenotype with 94% cells expressing Tie-2, 85% von Willebrand factor, and 66% eNOS. Our findings confirm the previous data by other investigators reporting that embryonic stem cells encapsulated in HA hydrogels remain capable of proliferating, maintain their undifferentiated state, and maintain their full differentiation capacity (11).

Next, we inquired whether HA hydrogel can retard the diffusion of LPS. HA hydrogels fabricated using increasing concentrations of the crosslinker were incubated for 90 min with LPS (5 µg/ml) conjugated with Alexa Fluor 594. At the end of incubation, brightfield and fluorescence images of hydrogels were acquired and analyzed for intensity of LPS fluorescence-penetrating scaffolds. While increasing the crosslinker PEGDA concentration did not reduce HA hydrogel pore size enough to completely abolish LPS diffusion into the hydrogel, diffusion was significantly reduced and embedded EPC viability was greatly enhanced when a higher percentage of crosslinker was used. As shown in Fig. 1, left, fabrication using 2% crosslinker was associated with a deeper diffusion along the centripetal axis to a depth of 250 µm. In contrast, at 4 and 8% crosslinker concentrations, penetration of HA hydrogels by LPS was substantially decreased.

These findings were further buttressed in experiments where EPCs were embedded into HA hydrogels prepared using 2, 4, and 8% crosslinker (PEGDA) and exposed to LPS for 24 h followed by a live/dead cell assay. As depicted in Fig. 1, right, EPCs showed an exponential gradient of dead cells from the periphery toward the center of HA hydrogels manufactured using 2% crosslinker, whereas the number of dead EPCs in HA hydrogels manufactured using 4 and 8% crosslinker was dramatically reduced. This finding was further corroborated when effects of LPS on embedded EPCs were compared with those observed in EPCs cultured on the surface of hydrogels. As shown in Fig. 2, the number of viable EPCs embedded in HA hydrogels fabricated using 4% crosslinker was relatively preserved despite the escalating concentrations of LPS. This was in contrast to the progressively increased proportion of dead cells cultured on the surface of hydrogels. Collectively, these data demonstrated that HA hydrogels utilizing 4% and higher concentrations of crosslinker reduced the penetration of LPS into these scaffolds and provided a sheltered microenvironment for embedded EPCs. Based on these findings, we used hydrogels fabricated with 4% crosslinker in the subsequent experiments.

The expression of TLR4, the mediator of LPS cytotoxicity, was examined using FACS analysis and immunocytochemistry staining of cultured EPCs. FACS analysis showed that 23% of viable cells expressed TLR4 (Fig. 3A), whereas immunocytochemistry analysis revealed 41.6% of cells express the receptor (Fig. 3, B-D). These findings provided an explanation for the observed vulnerability of EPCs exposed to LPS.

With this background information, we were able to address the main question: is adoptive transfer of EPCs capable of changing the course of endotoxemia and its effect on target organ function? CellTracker CM-Dil-labeled EPCs were embedded in HA hydrogels and implanted subcutaneously. Two days later, mice received intraperitoneal injection of LPS, followed within 1 h by either intravenous injection of EPCs or the release of embedded EPCs by digesting hydrogels with injection of hyaluronidase directly into hydrogel implants. Blood pressure was reduced in LPS mice, but cell therapy, regardless of the means of delivery, normalized it (Fig. 4A). Plasma ALT and AST levels were elevated in the LPS group, but adoptive transfer of EPCs corrected LPS-induced hepatotoxicity with HA hydrogel-embedded EPCs significantly more

**Fig. 5.** Plasma creatinine levels after either LPS injection (A) or cecal ligation and puncture (CLP; B). EPCs were delivered to endotoxemic mice either intravenously or by implantation of EPC containing hydrogels into the ear or kidney. Delivery of EPC, particularly by hydrogel, demonstrated significantly reduced creatinine levels. *P < 0.05 vs. LPS (hydrogel alone and no treatment). **P < 0.05 vs. control. †P < 0.05 vs. IV; n = 6.
effective than intravenous delivery (Fig. 4, B and C). Moreover, colony formation, severely impaired in LPS-treated mice, was significantly restored in LPS-treated mice receiving cell therapy (Fig. 4D).

To confirm the therapeutic benefits of HA hydrogel delivery of EPC occurred due to EPC themselves and not the HA hydrogel or its metabolites, HA hydrogels without embedded EPC were also implanted (and subsequently dissolved with digestive enzymes) in mice and used in experiments. In all experiments, implanted HA hydrogels without embedded EPC had no effect on any of the measured parameters. HA and its metabolites have previously been found to alter the septic response to LPS (23). However, the low level of HA within the 10 μl of implanted HA hydrogels (<0.8%) did not show any therapeutic efficacy in altering the septic response to LPS.

Renal function was chosen as a read-out system because of the sensitivity of the kidney to LPS (9). EPCs were implanted in ears or directly under the renal capsule. Two days later, mice received intraperitoneal injection of LPS, followed by release of HA hydrogel-embedded EPCs. [The release of subcapsularly implanted EPCs occurs due to the constitutive activity of hyaluronidase in the kidney, thus negating the need for exogenous hyaluronidase (8, 16, 18, 19, 32).] Plasma creatinine levels were significantly elevated in LPS-treated animals (Fig. 5A). Implantation of EPCs embedded in HA hydrogel resulted in a significantly improved renal function regardless of the site of implantation. Intravenous injection of the same number of EPCs also improved renal function, but had an intermediate efficacy.

These findings were corroborated by the results obtained from mice subjected to CLP, a model considered to better emulate polymicrobial human sepsis (29). CLP was associated with significant renal dysfunction, which was mitigated by adoptive transfer of EPCs (Fig. 5B). Our CLP model was associated with a mortality rate of 100% within 30 h of CLP-induced sepsis; however, survival was prolonged upon adoptive transfer of EPC. EPC delivery prolonged survival and resulted in a 10 and 30% survival rate through 60 h post-CLP when EPCs were delivered by intravenous or HA hydrogel implantation, respectively (n = 10).

In the next series of studies, we attempted to assess the remote consequences of EPC transplantation. In acute morphological analysis 18 h after LPS injection, kidneys and livers did not exhibit any significant morphological change and demonstrated only rare scattered neutrophilic infiltration. In long-term analysis, adoptive transfer of EPC significantly alleviated renal fibrosis observed by 2 mo after endotoxemia, especially upon delivery of EPC by HA hydrogel (Fig. 6). At 2 mo, EPC delivery by HA hydrogel was more effective in preventing fibrosis than conventional intravenous delivery.

Renal hemodynamics were examined in endotoxemic animals using LDF. Local hemodynamics, severely impaired in LPS-treated mice, were significantly restored in mice receiving EPCs embedded in HA hydrogel implants (Fig. 7). Intravenous injection of fibrosis 2 mo after endotoxemia (LPS) and treatment with EPC. Kidney sections were stained with Masson’s trichrome staining (A) 2 mo after LPS injection with or without adoptive transfer of EPC either by IV injection or HA hydrogel delivery. Hydrogels were also implanted without EPC (hydrogel alone). Renal fibrosis was quantified using digital image analysis (Image J program; B). *P < 0.05 vs. control. †P < 0.05 vs. EPC delivery (hydrogel and IV); n = 6. Magnification ×100.
administration of the equal number of EPCs also resulted in improved microcirculation, albeit with a lower efficacy.

As demonstrated above, adoptive transfer of EPC conferred resistance to endotoxemia irrespective of the way cells were delivered. Differences existed only in the efficacy of equivalent numbers of EPC, with the subcapsular and subcutaneous HA hydrogel delivery showing higher potency. The observed effect of EPC could not be attributed entirely to the engraftment of kidneys by EPC, as <1% of EPC engrafted the kidney after intravenous delivery. HA hydrogel delivery of EPC resulted in a fourfold increase in EPC engraftment when HA hydrogels were implanted in ears and an eightfold increase when they were implanted superficially in the kidney (Fig. 8). In either case, the overall number of engrafting cells was rather modest, thus casting doubt that this mechanism could explain the entire range of functional improvement.

The search for such alternative or additional factors revealed that adaptive transfer of EPC ameliorated systemic inflammatory response to the endotoxin. It has been previously established that mesenchymal stem cells exhibit a paracrine modulatory effect on cytokine expression (6, 25, 34, 39); however, no data exist on the ability of EPCs to modify the level of proinflammatory cytokines. The results of the multiplex profiling are summarized in Fig. 9. LPS greatly increased the levels of IFN-γ, IL-1, IL-6, and TNF-α, among others, and adoptive transfer of EPCs resulted in their decrease: levels of IFN-γ, GM-CSF, KC (IL-8), IP-10, IL-6, and IL-1β showed dramatic decline that may have contributed to EPC effects on renal function, renal blood flow, and systemic blood pressure. These findings argue in favor of paracrine effects of transplanted EPC in moderating systemic inflammatory response.

**DISCUSSION**

Data presented herein showed that EPCs are vulnerable to LPS and demonstrated the basis for this vulnerability-expression of TLR4 (Fig. 3). Furthermore, EPCs embedded in HA hydrogels were significantly protected from LPS cytotoxicity compared with two-dimensional cultures of the cells and the differential susceptibility can be explained by the diffusional constraints imposed by extensively crosslinked HA hydrogels (Figs. 1 and 2). Finally, we demonstrated that adoptive transfer of EPCs to mice with endotoxemia (induced by either LPS injection or CLP) afforded functional renoprotection, at least in part due to the improved renal microcirculation and moderating the cytokine storm (Figs. 7 and 9). Furthermore, the existing ambiguity related to the identity of murine EPC has been bypassed in our experiments by the use of an established embryonic EPC cell line.

Indeed, impaired renal hemodynamics has been found to be crucially involved in kidney injury during sepsis (9). TNF-α and LPS activate endothelial cells (36, 46), which undergo structural and functional changes including cellular detachment and denudation, altered vasomotor tone, and programmed cell death, which can lead to microvascular injury and a decrease in capillary perfusion, an important contributor to development of multi-organ failure in sepsis (30, 36). Although renal blood flow appeared to be increased in large animals (17), renal microcirculation was found to be diminished in others (37, 38, 43). These processes are opposed by restoration of endothelial integrity via migration and proliferation of mature endothelial cells (3, 27, 33) and/or recruitment of EPCs. Our LDF findings are in concert with these previous observations and demonstrated, for the first time, that adoptive transfer of EPC was associated with improved renal microcirculation. Comparative analysis of the modes of EPC delivery revealed that subcapsular renal delivery of EPCs embedded in HA hydrogel exerted a most profound effect, followed by the subcutaneous administration of embedded cells, whereas intravenous delivery, although also therapeutic, was the least efficacious (Fig. 7). Although these differences paralleled the differences in the number of engrafting EPC, a relatively low frequency of kidney-lodged cells suggests that this is not the major route responsible for increased renal microcirculation.
The reason for such a differential efficacy of equal numbers of cells (delivered embedded in HA hydrogels or intravenously as a suspension) may lie in the fact that these cells are vulnerable to LPS and that HA hydrogel, at least temporarily until its digestion, shields the cells from endotoxin. Recent clinical evidence suggested that stem and progenitor cells are affected in septic patients (25, 40). EPCs are reported to be mobilized during sepsis and play a pivotal role in re-endothelialization after vascular damage and severe inflammation (4, 5, 27, 40). For instance, studies by Mao et al. (20) demonstrated intravenous delivery of EPC attenuated LPS-induced lung injury including reduced pulmonary edema, hemorrhage, and inflammation. However, reports demonstrated that endotoxin can decrease EPC numbers by as much as 62% (21). Despite some discrepancies regarding the direct effect of sepsis on EPCs, clinical data show a remarkable correlation between patients’ survival rates and circulating EPCs during septic shock (4, 5, 27). In contrast, statin-induced increase in endogenous EPC mobilization during LPS did not reduce inflammation further highlighting the toxic effect of endotoxins on endogenous pools of EPC (31).

In this context, HA hydrogel represents a “honeycomb” structure with EPCs trapped within it. Hyaluronan is an intrinsic component of the extracellular matrix and stem cell niches, thus making hyaluronan ideal for inclusion when designing a scaffold aimed at mimicking endogenous stem cell niches. This scaffold is capable of impeding the penetration of LPS thus shielding the embedded cells from its cytotoxicity (Fig. 1). A similar shielding effect was observed in our previous study of adriamycin cytotoxicity (28). The release of EPCs occurs through the action of hyaluronidase/collagenase, which digest the scaffold without impairing the cells or inducing systemic effects per se (28). When HA hydrogels are implanted subcutaneously, the locally enhanced activity of these enzymes is sufficient to digest the scaffold and release the cells (16, 28). Some of the highly desirable therapeutic advantages of delivering cells embedded in HA hydrogel consist in 1) the ability to externally time their release and, even more remarkable, 2) the intrinsic capacity of the injured organ to regulate the release of EPCs from the implanted scaffold (organs such as the kidney constitutively release endogenous hyaluronidase) (8, 16, 18, 19, 32). The duration of a latent period before cells are released from the scaffold and become exposed to the circulating cytotoxins represents the major difference between the adoptive transfer of cells via hydrogel vs. intravenous delivery.

Our findings of impaired renal microcirculation in endotoxemic mice and its significant improvement (together with improved renal function and systemic blood pressure) after EPC delivery correlate with the concomitantly decreased levels of proinflammatory cytokines (Figs. 4A, 5, 7, 9). Xu et al. (39) demonstrated similar effects following adoptive transfer of mesenchymal stem cells. Our results show that levels of both...
pro- and anti-inflammatory cytokines are reduced through EPC delivery following LPS-induced endotoxemia (Fig. 9). The paracrine function of EPCs may mediate some of the reparative effects of these cells in the damaged kidney. Yasuda et al. (44) showed that intravenous injection of EPCs improved kidney function during adriamycin-induced nephropathy at least in part by reducing plasma cytokine levels. Our present findings also show that delivery of EPCs improves renal function in response to LPS. The secretome of the EPC has been previously studied using a combination of proteomic techniques, which revealed paracrine angiogenic factors like thymidine phosphorylase, MMP-9, IL-8, pre-B-cell enhancing factor, and macrophage migration inhibitory factor, among others (26). Our cytokine profiling following EPC delivery in endotoxemia shows that it modified numerous cytokines including IL-1β, IL-6, and IP-10 (Fig. 9) and that the reduced inflammation perhaps resulted in decreased IL-10 levels. The increase in the renoprotective effects of EPCs delivered by HA hydrogel, compared with intravenous delivery, during sepsis may in part be attributed to the increase in renal engraftment of the delivered EPCs, thus allowing for their enhanced paracrine effect on neighboring damaged cells.

On a broader scale, these studies support the role of microcirculatory failure as a critical contributor to septic AKI and indicate that EPC-based cell therapy effectively preserves renal microcirculation in a murine model. However, exposure to
circulating toxins can directly damage both the endogenous and the delivered EPCs. The data indicate that therapeutic benefits of the adoptive transfer of EPCs embedded in HA hydrogels are superior to those of intravenously delivered cells in a murine model of sepsis.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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