Resident Endothelial Cells and Endothelial Progenitor Cells Restore Endothelial Barrier Function After Inflammatory Lung Injury

Sun-Zhong Mao,* Xiaobing Ye,* Gang Liu, Dongmei Song, Shu Fang Liu

Objective—Disruption of endothelial barrier integrity is a characteristic of many inflammatory conditions. However, the origin and function of endothelial cells (ECs) restoring endothelial barrier function remain unknown. This study defined the roles of resident ECs (RECs) and bone marrow–derived endothelial progenitor cells (BMDEPCs) in endothelial barrier restoration after endotoxemic lung injury.

Approach and Results—We generated mice that enable to quantify proliferating RECs or BMDEPCs and also to study the causal link between REC or BMDEPC proliferation and endothelial barrier restoration. Using these mouse models, we showed that endothelial barrier restoration was associated with increased REC and BMDEPC proliferation. RECs and BMDEPCs participate in barrier repair. Immunofluorescence staining demonstrated that RECs proliferate in situ on endothelial layer and that BMDEPCs are engrafted into endothelial layer of lung microvessels at the active barrier repair phase. In lungs, 8 weeks after lipopolysaccharide-induced injury, the number of REC-derived ECs (CD45<sup>-</sup>/CD31<sup>+</sup>/BrdU<sup>+</sup>/rtTA<sup>+</sup>) or BMDEPC-derived ECs (CD45<sup>-</sup>/CD31<sup>+</sup>/eNOS<sup>+</sup>/GFP<sup>+</sup>) increased by 22- or 121-fold, respectively. The suppression of REC or BMDEPC proliferation by blocking REC or BMDEPC intrinsic nuclear factor-κB at the barrier repair phase was associated with an augmented endothelial permeability and impeded endothelial barrier recovery. RECs and BMDEPCs contributed differently to endothelial barrier repair. In lungs, 8 weeks after lipopolysaccharide-induced injury, REC-derived ECs constituted 22%, but BMDEPC-derived ECs constituted only 3.7% of the total new ECs.

Conclusions—REC is a major and BMDEPC is a complementary source of new ECs in endothelial barrier restoration. RECs and BMDEPCs play important roles in endothelial barrier restoration after inflammatory lung injury. (Arterioscler Thromb Vasc Biol. 2015;35:1635-1644. DOI: 10.1161/ATVB.AHA.115.305519.)

Key Words: endothelial cells ■ NF-κB

Disruption of endothelial barrier and increase in endothelial permeability are major features of acute lung injury (ALI) associated with sepsis, trauma, and hemorrhage. However, the mechanisms regulating endothelial barrier restoration are poorly understood. Previous studies showed that fox head box M1-regulated proliferation genes play important roles. However, the effector cells that mediated the repair function of fox head box M1 have not been identified. The origin and function of endothelial cells (ECs) in endothelial barrier restoration after inflammatory organ injury remain unknown.

Resident ECs (RECs) are long believed to be a major source of ECs in endothelial repair. Indeed, neighboring RECs were observed to sprout into denuded area after mechanical arterial endothelial denudation. However, the role of RECs in the restoration of endothelial barrier function after organ injury has not been studied. A causal link between REC proliferation and endothelial barrier restoration remains to be established. Bone marrow–derived endothelial progenitor cells (BMDEPCs) as a source of ECs in angiogenesis and endothelial repair have been extensively studied, although whether BMDEPCs contribute to endothelial or vascular wall is controversial. Depending on type, nature, and severity of the injury, BMDEPCs were reported to contribute to endothelial and vascular repair in some experimental models but play no role in other models. Microvascular injury associated with septic ALI differs significantly from models used in previous reports in nature and severity. The contribution of BMDEPCs to endothelial barrier repair in septic organ injury is still unclear. Increased EPC mobilization and recruitment were observed in patients and animal models with septic organ injury. Autologous transplantation of EPCs suppressed lung inflammation, attenuated endothelial permeability, and lung edema and improved outcomes. However, it remains unclear whether the exogenous EPCs improve endothelial barrier function by preventing endothelial injury or by promoting endothelial repair, or by both. Other investigators have demonstrated that exogenously administered EPCs or other stem/progenitor cells did not participate in endothelial repair.
Nonstandard Abbreviations and Acronyms

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<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>ALI</td>
<td>acute lung injury</td>
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<td>BMDEPCs</td>
<td>bone marrow–derived endothelial progenitor cells</td>
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<td>BrdU</td>
<td>bromodeoxyuridine</td>
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<td>EC</td>
<td>endothelial cell</td>
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<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
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<td>FACS</td>
<td>fluorescence activated cell sorting</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<td>MLC</td>
<td>myosin light chain</td>
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<td>NF-κB</td>
<td>nuclear factor-κB</td>
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<td>REC</td>
<td>resident ECs</td>
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<tr>
<td>rtTA</td>
<td>reverse tetracycline transactivator</td>
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but alleviated organ injury and improved outcomes by immune-modulating and paracrine mechanisms.\(^{22–24}\) Furthermore, no previous study has examined the causal link between BMDEPC recruitment/proliferation and endothelial barrier restoration. Thus, whether BMDEPC is a source of ECs in endothelial barrier restoration remains unclear.

To study the causal link between REC or BMDEPC proliferation and endothelial barrier restoration, we need to examine the functional effect of inhibiting REC or BMDEPC proliferation on endothelial barrier restoration. In addition, barrier injury and repair are inter-related. Severity of injury determines the extent of repair. It is ideal to inhibit REC or BMDEPC proliferation only at the barrier repair phase. Animal model that enables to selectively inhibit REC or BMDEPC proliferation at the barrier repair phase are needed. No such an animal model has been reported.

In this study, we took advantage of the fact that endothelial repair depends mainly on proliferation of EC precursor cells and that the nuclear factor-κB (NF-κB) pathway is a major pathway controlling cell proliferation.\(^{25–30}\) We created EC-IκBα-WT-BM or WT-EC-IκBα-BM chimeric mice with doxycycline-inducible and REC- or BMDEPC-restricted overexpression of a mutant IκBα (IκBαmt). By treating these mice with doxycycline after peak of lung injury, we were able to inhibit REC or BMDEPC proliferation at the barrier repair phase by blocking REC or BMDEPC intrinsic NF-κB activity in a cell-targeted and stage-specific manner. By performing cause-to-effect studies in combination with cell fate mapping, we demonstrated that REC is a major and BMDEPC is a complementary source of new ECs in endothelial barrier repair and that RECs and BMDEPCs play important roles in endothelial barrier restoration after inflammatory ALI.

### Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

### Results

**Active Endothelial Barrier Repair Occurs at 48 Hours**

Endothelial permeability increased progressively between 0 and 24 hours, associated with increasing numbers of apoptotic ECs, and decreased progressively between 24 and 96 hours, associated with decreasing numbers of apoptotic ECs and increasing numbers of proliferating ECs (Figure 1A).\(^{30}\) On the basis of these observations, we defined 0 to 24 and 24 to 96 hours, respectively, as endothelial barrier injury and repair phases. At 48 hours, endothelial permeability decreased rapidly, associated with the highest level of EC proliferation (Figure 1A).\(^{30}\) We considered 48 hours as the active barrier repair phase and focused our subsequent studies on 48 hours.

#### Endothelial Barrier Recovery Is Associated With Increased REC and BMDEPC Proliferation

If RECs and BMDEPCs play important roles in endothelial barrier repair, the number of proliferating RECs or BMDEPCs should increase at the active repair phase. To track proliferating RECs or BMDEPCs in vivo, we generated EC-rtTA-GFP-BM chimeras by transplanting lethally irradiated EC-rtTA mice\(^{31}\) with bone marrows (BMs) from Tie2-GFP mice (Tables I and II in the online-only Data Supplement). These chimeras overexpress the reverse tetracycline transactivator (rtTA) on RECs and green fluorescent protein (GFP) on BMDEPCs. Fluorescence-activated cell sorting (FACS) analysis of BM mononuclear cells from donors and chimeras 2 months later confirmed that 95% of BM cells in the chimeras were donor BM origin (Figure 1A in the online-only Data Supplement).

![Figure 1. Endothelial barrier recovery is associated with increased resident endothelial cell (REC) and bone marrow–derived endothelial progenitor (BMDEPC) proliferation.](image-url)
FACS analysis of peripheral blood mononuclear cells further confirmed the high degree of BM chimerism in the EC-rtTA-GFP-BM mice (data not shown).

The endothelial-specific Tie2 promoter drives GFP expression in endothelial lineage cells. However, Tie2 was reported to be expressed on a subset of monocytes/macrophages. To clarify the percentage of monocytes in the GFP+ cell population in the lungs, we phenotyped GFP+ cells from lungs of Tie2-GFP donor mice 48 hours after lipopolysaccharide challenge. We found that 99% of the GFP+ cells are CD45+/CD31+/eNOS+ endothelial lineage cells, and <1% of them are CD45+/CD31+ hematopoietic cells (Figure II in the online-only Data Supplement). Our finding is consistent with a previous report. Thus, GFP+ cells in the EC-rtTA-GFP-BM mice represent principally BMDEPCs.

Two months after BM transplantation, mice were injected with saline or lipopolysaccharide and then with bromodeoxyuridine (BrdU) to label proliferating cells in vivo. Proliferating RECs or BMDEPCs, or recruited BMDEPCs, were identified by BrdU/rtTA or BrdU/GFP double staining, or by GFP staining, and counted. Time-course analyses showed that endothelial barrier recovery was associated with remarkably increased REC and BMDEPC proliferation (Figure 1). In particular, the high numbers of proliferating RECs and BMDEPCs at 48 hours were concordant with 48 hours being active barrier repair phase. No significant numbers of proliferating RECs and BMDEPCs were detected at 12 (injury phase) or 96 hours (barrier function recovered; Figure 1). ALI was associated with an increased recruitment of GFP+ BMDEPCs in lungs (Figure 1D). However, BMDEPC recruitment was not correlated with barrier recovery but correlated with barrier injury (Figure 1A versus 1D), suggesting that ALI stimulates BMDEPC recruitment.

**RECs Participate in Endothelial Barrier Repair**

If RECs participate in endothelial barrier repair, these cells should proliferate in situ on endothelial layer at the active repair phase to give rise to new ECs. Furthermore, the REC-derived daughter ECs should significantly increase in lungs after recovery from injury. EC-rtTA-GFP-BM mice that overexpress rtTA only on RECs (Table II in the online-only Data Supplement) were injected with BrdU at 44 hours after lipopolysaccharide injection to label proliferating cells. Lungs were harvested at 48 hours or at 8 weeks after lipopolysaccharide injection to track the location of proliferating RECs or to quantify the REC-derived new ECs in lungs. We visualized endothelial layer by immunofluorescence staining of lung sections with rtTA or CD31 antibody. We identified proliferating RECs by BrdU and rtTA double immunofluorescence staining. Confocal microscopic examination revealed that BrdU+/rtTA+ proliferating RECs were localized on the endothelial layer of microvessels (Figure 2A). The BrdU+/rtTA+ proliferating RECs coexpressed EC marker, CD31, and were localized on the CD31+ endothelial layer but were not localized on the aquaporin-5 (Aq5)+ epithelial layer (Figure 2A). This result provides histological evidence that RECs proliferate in situ on endothelial layer at the active barrier repair phase.

FACS analysis showed that the number of REC-derived new ECs (CD45−/CD31+/BrdU+/rtTA+) was ≈22-fold higher in lungs of EC-rtTA-GFP-BM mice 8 weeks after lipopolysaccharide-induced injury, compared with lungs from mice 8 weeks after saline injection (Figure 2B and 2C). These results provide cytological evidence for REC’s participation in endothelial barrier repair.

**BMDEPCs Contribute to Endothelial Barrier Repair**

BMDEPC incorporation into endothelial layer is a critical step in BMDEPC-mediated endothelial repair. To seek histological evidence of BMDEPC engraftment, we stained lung sections from mice 48 hours after lipopolysaccharide injection with antibodies against BMDEPC marker, GFP, EC markers, CD31 and Ve-cadherin (Ve), or alveolar epithelial cell marker, Aq5. Confocal microscopic examination identified GFP+/CD31+ BMDEPCs localized on the CD31+ endothelial layer of lung microvessels (Figure 3A). The GFP+ BMDEPCs were also Ve+ and localized on the Ve+ endothelial layer but not localized on the Aq5+ alveolar epithelial layer (Figure 3A). This result provides evidence for BMDEPC engraftment into the endothelial layer in the lung at the active repair phase.

We quantified the engrafted BMDEPCs (CD45−/CD31+/eNOS+/GFP+) in lungs from mice 8 weeks after saline or lipopolysaccharide injection, a time point when organ inflammation and inflammation-associated BMDEPC recruitment has been subsided. FACS analysis showed that the number of CD45−/CD31+/eNOS+/GFP+ cells was ≈121-fold higher in lipopolysaccharide-injected than in saline-injected lungs (Figure 3B and 3C), indicating an increased BMDEPC engraftment in injured lungs. These results provide cytological evidence for BMDEPC engraftment into lung microvessels and suggest that BMDEPC contribute to endothelial barrier repair.

**Different Contributions of RECs Versus BMDEPCs to Endothelial Barrier Repair**

We next compare the relative contributions of RECs versus BMDEPCs to endothelial barrier repair. We counted the numbers of proliferating RECs and BMDEPCs in lungs at 48 hours and the numbers of REC- and BMDEPC-derived new ECs in lungs 8 weeks after lipopolysaccharide-induced injury. At 48 hours, the number of proliferating RECs was 2.4-fold higher than proliferating BMDEPCs in lipopolysaccharide-challenged lungs, although both proliferating RECs and BMDEPCs were remarkably higher than control lungs (Figure 4A and 4B). In lungs, 8 weeks after lipopolysaccharide-induced injury, total number of new ECs, defined as CD45−/CD31+/BrdU+ cells, accounted for ≈2% of total lung cells, which is in agreement with our previous observation that apoptotic ECs accounted for ≈2% of total lung cells in this mouse model of ALI. Among the new EC subpopulation, 83% were rtTA+ (REC-derived), but only 9.5% were GFP+ (BMDEPC-derived; Figure 4C and 4D).

BMDEPCs may replace apoptotic/dead ECs by differentiation. We compared the number of REC-derived ECs (CD45−/CD31+/BrdU+/rtTA+ cells) to the number of total engrafted BMDEPCs (CD45−/CD31+/eNOS+/GFP+ cells), which includes BrdU+ and BrdU−, BMDEPC-derived ECs, in lungs of EC-rtTA-GFP-BM mice (Table II in the online-only
Data Supplement) 8 weeks after lipopolysaccharide injection. REC-derived ECs constituted 22%, but the total engrafted BMDEPCs constituted only 3.7% of the total CD45−/CD31+ EC population (Figure 4E). Thus, both histological and FACS analyses revealed that RECs are a major source and BMDEPCs is a complementary source of new ECs in endothelial barrier repair in the lungs.

Targeted Inhibition of REC or BMDEPC Intrinsic NF-κB Activity Suppresses REC or BMDEPC Proliferation

We previously demonstrated that doxycycline-induced I-κBαmt expression inhibited NF-κB in endothelial lineage cells but not in other cell types in the EC-I-κBαmt mice.31 Using the EC-I-κBαmt mice as recipients or donors, we generated EC-I-κBα-WT-BM or WT-EC-I-κBα-BM chimeras (Table II in the online-only Data Supplement). We confirmed the high level of donor BM engraftment (>95%) in the chimeras (Figure IB and IC in the online-only Data Supplement). EC-I-κBα-WT-BM or WT-EC-I-κBα-BM mice overexpress I-κBαmt on RECs or BMDEPCs and enabled us to selectively block REC or BMDEPC intrinsic NF-κB activity through doxycycline-induced I-κBαmt expression, which leads to the suppression of REC or BMDEPC proliferation. At 48 hours post lipopolysaccharide, tissue NF-κB activity is reduced to a low level. Techniques (immunofluorescence and immunohistochemistry) capable of revealing EC-selective NF-κB inhibition are not sensitive enough to

Figure 2. Resident endothelial cells (RECs) participate in endothelial repair. A, RECs proliferate in situ on the endothelial layer at the active repair phase. Lung sections from mice 48 hours after lipopolysaccharide (LPS) injection were stained with antibodies against proliferative marker, 5-bromo-2-deoxyuridine (BrdU), REC marker, reverse tetracycline transactivator (rtTA), EC marker, CD31, and alveolar epithelial cell marker, aquaporin-5 (Aqu5), and nuclei counterstained with TO-PRO-3 dye (Pro-3). 3D projections (A1–A6) or single images (A7–A10) of confocal z-stacks are shown. A1, BrdU-staining (green) detects proliferating cells (light blue nuclei); blue, Pro-3 nuclear staining. A2, rtTA-staining (red) detects RECs and visualizes the endothelial layer. A3, Merge of A1 and A2 shows BrdU/rtTA REC (arrow indicated) localized on rtTA endothelial layer of alveolar microvessels. A4 and A5, Orthogonal view (X–Y, X–Z, and Y–Z) of the boxed area in A3 at higher magnification confirms colocalization of BrdU and rtTA signals and colocalization of BrdU and Pro-3–stainings. Note, the blue nuclear staining in A4 or the red rtTA staining in A5 was omitted for clarity. A6 and A7, BrdU+/CD31+ RECs (arrow indicated) are localized on CD31 endothelial layer of alveolar microvessels. A8–A10, Higher magnification of the boxed area in A7 is shown. A8, BrdU (green) and CD31 (red) double stain shows that BrdU proliferating REC is localized on CD31 endothelial layer (red). A9, BrdU (green) and Aqu5 (blue) double stain shows that BrdU proliferating REC is not localized on Aqu5 epithelial layer (blue). A10, Merge of A8 and A9 confirms that BrdU REC is localized on the endothelial layer (red) between 2 epithelial layers (blue). Scale bars, 40 µm (A1, A2, A3, A6 and A7), 8 µm (A4 and A5), and 3 µm (A8, A9, and A10). Fluorescence-activated cell sorting pictures (B) and bar graph (C) show an increased number of REC-derived ECs, defined as CD45+/CD31+/rtTA+/BrdU+ cells, in lungs of mice 8 weeks after LPS injection, compared with saline-injected mice (Con). Mean±SEM of 5 mice per group. *P<0.05, compared with control.
detect the subtle change in NF-κB activity caused by doxycycline-induced I-κBm expression. We verified that treatment of the chimeras with doxycycline induces I-κBm expression and inhibits NF-κB activity. We showed that injection of EC-I-κBα-WT-BM mice with doxycycline at 36 hours post lipopolysaccharide induced high level of I-κBm mRNA expression (Figure III in the online-only Data Supplement) and repressed lung tissue level of vascular cell adhesion molecule protein, a widely used marker of endothelial NF-κB activity, at 48 hours (Figure 5A).

Targeted inhibition of REC or BMDEPC intrinsic NF-κB activity suppressed REC or BMDEPC proliferation. At 48 hours after lipopolysaccharide and 12 hours after doxycycline injection, the number of proliferating RECs or BMDEPCs in lung sections of EC-I-κBα-WT-BM or WT-EC-I-κBα-BM mice (Table II in the online-only Data Supplement, with NF-κB inhibition) was significantly lower than that in lung sections of EC-rtTA-GFP-BM or WT-EC-rtTA-BM mice (Table II in the online-only Data Supplement, without NF-κB inhibition; Figure 5B and 5C).

Suppression of REC or BMDEPC Proliferation at Active Repair Phase Was Associated With an Augmented Endothelial Permeability

At 48 hours after lipopolysaccharide and 12 hours after doxycycline injection, wild-type (WT) mice, in which REC or BMDEPC proliferation was not inhibited, displayed a moderate increase in endothelial permeability that is consistent with endothelial barrier repair phase (Figure 5D). EC-I-κBα-WT-BM or WT-EC-I-κBα-BM mice, in which REC or BMDEPC proliferation was inhibited, exhibited a significantly augmented endothelial permeability (Figure 5D). Blockade of
REC intrinsic NF-κB activity caused a 42% reduction in REC proliferation and 41% augmentation of endothelial permeability in the EC-I-xBα-WT-BM mice (Figure 5B versus 5D), suggesting that augmentation of endothelial permeability and suppression of REC proliferation are causally related.

**Proliferation-Independent Mechanisms Do Not Contribute to the Augmented Endothelial Permeability Caused by Endothelial NF-κB Blockade at 48 Hours**

In addition to controlling EC proliferation, NF-κB mediates endothelial permeability by inducing inflammatory gene expression and by disrupting interendothelial junctions, as results of myosin light chain (MLC) phosphorylation and junction protein internalization and downregulation. To clarify whether these proliferation-independent mechanisms may contribute to the augmented endothelial permeability caused by endothelial NF-κB blockade at 48 hours, we compared lung tissue levels of NF-κB–regulated cytokines, tumor necrosis factor-α, interleukin-1β, interleukin-6, and chemokine (C-X-C motif) ligand 1, between mice at 6 and 48 hours to assess organ inflammatory status at 48 hours, and between WT and EC-I-xBαmtm mice to examine the effects of endothelial NF-κB blockade. We used EC-I-xBαmtm mice (Table I in the online-only Data Supplement), the recipients or donors of EC-I-xBα-WT-BM or WT-EC-I-xBα-BM chimeras (Table II in the online-only Data Supplement), for these studies. The use of EC-I-xBαmtm mice enabled us to evaluate the effects of inhibiting REC and BMDEPC intrinsic NF-κB activities simultaneously. We also compared tissue levels of phospho–MLC-2 and membrane-bound VE-cadherin, 2 major markers of disruption of interendothelial junctions, between the 2 time points and the 2 groups of mice.

Lung tissue levels of the 4 cytokines at 48 hours were higher than that of controls but were many-fold lower than that at 6 hours, and, importantly, they were not affected by EC-restricted NF-κB inhibition (Figure IVA–IVD in the online-only Data Supplement). At 48 hours post lipopolysaccharide injection, lung tissue levels of phospho–MLC-2 and membrane-bound VE-cadherin proteins were all at control levels and were not affected by EC-restricted NF-κB inhibition (Figure IVF and Figure V in the online-only Data Supplement). By contrast, the lung tissue level of phospho–MLC-2 was several-fold higher at 3 hours, and the level of membrane-bound or cytoplasmic VE-cadherin protein was several-fold lower or higher at 48 hours than controls and at 48 hours, indicating increased MLC-2 phosphorylation and VE-cadherin internalization at 6 hours but not at 48 hours. All these changes were abrogated by EC-restricted NF-κB blockade (Figure IVF and Figure V in the online-only Data Supplement). This result excludes the possible involvement of NF-κB–mediated, proliferation-independent mechanisms in the augmentation of endothelial permeability caused by endothelial NF-κB blockade at 48 hours.
First, what are the origins of ECs in endothelial barrier repair after inflammatory organ injury. This study addresses 3 fundamental questions that are critical for the understanding of cellular mechanisms of endothelial barrier recovery and restoration: We demonstrated for the first time that both RECs and BMDEPCs are important sources of new ECs in endothelial barrier restoration. The recovery of endothelial barrier function was associated with a remarkably increased REC proliferation and increased BMDEPC proliferation and engraftment. Lungs at active barrier repair phase had the highest level of REC or BMDEPC proliferation and increased BMDEPC engraftment. In lungs, 8 weeks after lipopolysaccharide-induced injury, numbers of REC- and BMDEPC-derived ECs increased by 22- and 121-fold, respectively. More importantly, suppression of REC or BMDEPC proliferation at the active barrier repair phase (48 hours) was associated with an augmented endothelial permeability and impeded endothelial barrier recovery.

Second, what role does each EC precursor cell play in endothelial barrier repair? We showed that although both RECs and BMDEPCs participate in endothelial barrier repair, their quantitative contributions differ. In lungs at the active barrier repair phase, the number of proliferating RECs was more than double of proliferating BMDEPCs. In lungs, 8 weeks after lipopolysaccharide-induced injury, REC-derived ECs constituted 22%, but BMDEPC-derived ECs constituted only 3.7% of the total CD45+/CD31+ ECs. This result illustrates that REC is a major and BMDEPC is a complementary source of new ECs in endothelial barrier restoration after inflammatory ALI.

RECs and BMDEPCs also play different roles in maintaining normal endothelium. In lungs of mice 8 weeks after saline injection, we detected 1.07% REC-derived new ECs (CD45+/CD31+/rtTA+/BrdU+) but only 0.03% BMDEPC-derived ECs (CD45+/CD31+/eNOS+/GFP+) in the EC population (CD45+/CD31+), suggesting that maintenance of normal endothelium depends mainly on proliferation of RECs with minimal contribution by BMDEPCs. This result is in agreement with a previous report showing that maintenance of lung endothelium does not involve BMDEPCs.15

Third, is there a causal link between REC or BMDEPC proliferation and endothelial barrier restoration? This is the most important question that no previous study has attempted to address because of technical challenges. Cell proliferation is regulated by many signaling pathways and numerous cell cycle regulators with overlapping functions.37,38 The redundancy in the regulatory mechanisms makes it extremely difficult to achieve a clear-cut and high-level inhibition of REC or BMDEPC proliferation by targeting any single proliferation pathway. In addition, many of the proliferation-regulating pathways also regulate other biological processes. This compromises the selectivity in inhibiting cell proliferation. We may not be able to completely overcome the technical hurdles using currently available animal models but have to find a way to address this important question. In this study, we used REC- or BMDEPC-restricted inhibition of NF-κB activity as a means of suppressing REC or BMDEPC proliferation. We did so for 3 reasons: first, the NF-κB pathway is a major signaling pathway controlling cell proliferation; second, animal models are available in our laboratory;15 and third, inhibition of NF-κB–mediated EC proliferation and

**Discussion**

This study addresses 3 fundamental questions that are critical for the understanding of cellular mechanisms of endothelial barrier restoration after inflammatory organ injury. First, what are the origins of ECs in endothelial barrier repair and restoration?
inhibition of NF-κB–mediated, proliferation-independent mechanisms have opposite effects on endothelial permeability, which allow us to reliably assess the effect of inhibiting REC or BMDEPC proliferation on endothelial barrier restoration. We found that inhibition of REC or BMDEPC intrinsic NF-κB activity repressed REC or BMDEPC proliferation by 42% or 44%, implicating the important role of NF-κB pathway in controlling REC and BMDEPC proliferation and illustrating the effectiveness of blocking NF-κB pathway in repressing REC or BMDEPC proliferation.

Suppression of REC or BMDEPC proliferation by REC- or BMDEPC-restricted NF-κB blockade at 48 hours was associated with an augmented endothelial permeability. We interpret this augmentation, at least partially, as a consequence of suppressing REC or BMDEPC proliferation. EC apoptosis is a major mechanism underlying the higher endothelial permeability at 48 hours post lipopolysaccharide. Replacement of the apoptotic/dead ECs through proliferation of endothelial precursor cells (RECs and BMDEPCs) is critical to the restoration of endothelial barrier function. When REC or BMDEPC proliferation is repressed, particularly at the active barrier repair phase, the process of endothelial barrier repair is impeded and endothelial permeability increased. In supporting this contention, we demonstrated here that selective blockade of REC or BMDEPC intrinsic NF-κB activity at 48 hours suppressed REC or BMDEPC proliferation and concomitantly augmented endothelial permeability. Others have demonstrated a causal link between EC apoptosis and increased endothelial permeability.

Our previous study showed that inhibition of EC apoptosis ameliorated the augmentation of endothelial permeability caused by endothelial NF-κB blockade at 48 hours in this ALI model. In addition to controlling EC proliferation, NF-κB mediates lipopolysaccharide-induced endothelial permeability by inducing inflammatory gene expression, which causes the disruption of interendothelial junctions as results of MLC phosphorylation, EC contraction, and junction protein cytosolic translocation. However, these mechanisms are unlikely to contribute to the augmented endothelial permeability caused by REC- or BMDEPC-targeted NF-κB inhibition at 48 hours. At this stage, tissue levels of NF-κB–regulated cytokines have become low and were not affected by endothelial NF-κB blockade. Tissue levels of phospho-MLC and membrane-bound VE-cadherin, 2 major markers of disruption of interendothelial junctions, were at control levels, suggesting that the lipopolysaccharide-induced disruption of interendothelial junctions has been restored at this time point. More importantly, it is well documented that inhibitions of NF-κB–mediated inflammatory cytokine expression and NF-κB–mediated disruption of interendothelial junctions decrease but not increase endothelial permeability.

Other factors may also contribute to the augmentation. NF-κB blockade in BMDEPCs may inhibit BMDEPC migration, adhesion, and incorporation, which can impede BMDEPC-mediated barrier repair. However, this mechanism may not contribute to the augmented endothelial permeability caused by REC-selective NF-κB blockade. Neighboring REC4 replace apoptotic/dead ECs by proliferating in situ on the endothelial layer, and by sprouting toward the injured site, a process that may not involve significant cell migration and incorporation. To restore endothelial barrier function, the newly generated ECs need to interact with the existing ECs on the endothelial layer to re-establish normal interendothelial junctions. The effects of NF-κB blockade on these cell–cell interactions warrant further investigation. Collectively, these results suggest that suppression of REC or BMDEPC proliferation explains, at least partially, the augmented endothelial permeability caused by blockade of REC or BMDEPC intrinsic NF-κB activity at 48 hours, implying a causal relationship between REC or BMDEPC proliferation and endothelial barrier restoration.

We demonstrated previously that endothelial NF-κB blockade at 6 hours inhibited lipopolysaccharide-induced endothelial permeability but showed here that REC- or EPC-targeted NF-κB inhibition at 48 hours augmented endothelial permeability. This discrepancy could be explained by different biological function that NF-κB plays at the 2 time points. At 6 hours, NF-κB mediates the increased endothelial permeability by disrupting interendothelial junctions without involving significant EC apoptosis. Endothelial NF-κB blockade at this stage abrogated the disruption of interendothelial junctions but had little effect on EC apoptosis, resulting in a reduced endothelial permeability. At 48 hours, the disrupted interendothelial junctions have been fully restored and EC apoptosis has become a predominant mechanism underlying the increased endothelial permeability. Endothelial NF-κB blockade at this stage inhibited NF-κB–mediated EC proliferation and promoted EC apoptosis, both of which impeded barrier recovery and augmented endothelial permeability. Consistent with this explanation, we showed here that the tissue level of phospho-MLC or membrane-bound VE-cadherin was remarkably high or low at 6 hours but was at the control level at 48 hours. Endothelial NF-κB blockade abrogated lipopolysaccharide-induced increase in the tissue level of phospho-MLC, decrease in the tissue level of membrane-bound VE-cadherin, and increase in VE-cadherin internalization at 6 hours, but it had no effect at 48 hours. Our previous study showed that endothelial NF-κB blockade at 48 hours enhanced EC apoptosis and concomitantly augmented endothelial permeability. Others have demonstrated a causal link between EC apoptosis and increased endothelial permeability. Although the number of apoptotic/dead ECs is only a small fraction of total ECs, EC apoptosis/death leads to the formation of pores on endothelium, which dramatically increases endothelial leakiness.

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Disruption of endothelial barrier integrity and increase in endothelial leakiness are hallmarks of acute lung injury and other inflammatory conditions. However, the origin and function of new endothelial cells in endothelial barrier repair and restoration are unknown. Using novel transgenic mouse models, this study identifies resident endothelial cells as a major source and bone marrow–derived endothelial progenitor cells as a complementary source of new endothelial cells in endothelial barrier repair and restoration. We provide the first evidence that resident endothelial cells and bone marrow–derived endothelial progenitor cells play important functions in endothelial barrier restoration and demonstrate a causal relationship between resident endothelial cell or bone marrow–derived endothelial progenitor cell proliferation and endothelial barrier restoration. Our data provide important new insights into cellular mechanisms of endothelial barrier repair and restoration.
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SUPPLEMENTAL MATERIAL

Materials and Methods

Animal models:

The characterization of the EC-I-κBαm mice with endothelial cell (EC)-restricted and Dox-inducible overexpression of I-κBαm, and of the EC-rtTA mice with constitutive overexpression of the reverse tetracycline transactivator (rtTA) on ECs have been previously described in detail (Supplemental Table I). The Tie2-GFP mice that overexpress green fluorescent protein (GFP) on ECs were purchased from Jackson Laboratory. We used wild type (WT) and 4 chimeric mouse strains, EC-rtTA-GFP-BM, EC-I-κBα-WT-BM, WT-EC-rtTA-BM and WT-EC-I-κBα-BM mice for our studies (Supplemental Table II). All animal experiments were approved by institutional animal care and use committee and complied with NIH Guide for the care and use of laboratory animals. EC-rtTA-GFP-BM or EC-I-κBα-WT-BM chimera was created by transplanting lethally irradiated EC-rtTA or EC-I-κBαm mice with bone marrow (BM) from Tie2-GFP or WT mice. WT-EC-rtTA-BM or WT-EC-I-κBα-BM chimera was created by transplanting lethally irradiated WT mice with BM from EC-rtTA or EC-I-κBαm mice.

Mice were injected with doxycycline (Dox, 0.5 mg/mouse, i.p.) 36 hours after LPS injection, which induced I-κBαm expression in resident endothelial cells (RECs) or BM-derived endothelial progenitor cells (BMDEPCs) at 48 hours (active repair phase). This Dox treatment regimen avoids the confounding effect caused by NF-κB inhibition at endothelial injury phase. To avoid Dox effect, mice in control groups were also treated with Dox in same manner. To confirm that this Dox treatment regimen efficiently induces I-κBαm expression at desired time point, lungs were harvested from saline or Dox treated mice, and tissue level of I-κBαm mRNA determined using RT-PCR as we have previously described. To confirm that Dox-induced
I-κBαmt expression at repair phase inhibits REC intrinsic NF-κB activity, two groups of EC-rtTA-GFP-BM and EC-I-κB-WT-BM mice were injected with saline or LPS, and then with Dox (0.5 mg/mouse, i.p.) 36 hours later. Lungs were harvested 48 hours after LPS injection and tissue level of vascular cell adhesion molecule (VCAM)-1 protein determined using Western blot.

Bone marrow transplant:

Recipient mice (6-8 weeks) received a lethal dose of whole body X-ray irradiation (900 Rads) and transplanted with donor BM cells. Donor mice were euthanized. The femurs and tibias were removed aseptically and marrow cavities flushed with Ca++, Mg++-free Hanks’ Balanced Salt Solution. Single cell suspension was prepared, washed twice, counted, and resuspended in sterile RPMI medium at 3x10^7 cells/ml prior to transplantation. Each irradiated recipient was injected with 1x10^7 BM cells through tail vein.

Two months after BM transplantation, the chimeras were used for experiments or tissue collection. Reconstitution of recipient BM with donor BM cells was confirmed and degree of donor BM chimerism analyzed by fluorescence activated cell sorting (FACS) analysis. BM or peripheral blood mononuclear cells were isolated from donors, recipients and chimeras by Ficoll–hypaque density centrifugation and analyzed using FACS to determine the percentage of GFP+ or rtTA+ EPCs in BM or peripheral blood mononuclear cell population. Degree of donor BM chimerism was determined by comparing the percentage of GFP+ or rtTA+ EPCs between each chimera and its respective donor.

Assessment of endothelial permeability:

Microvascular endothelial permeability was assessed using Evans blue dye (EBD) leakage index as a marker, as previously described. To characterize the time course of endothelial barrier injury and repair, WT mice were injected with saline (controls) or Escherichia coli LPS (0111:B4, 5 mg/kg, i.p.), and endothelial permeability measured 12, 24, 48 or 96 hours after LPS injection. Mice were injected with EBD (20 mg/kg, i.v.) and then with heparin (200 U,
i.v.) at 1.5 and 0.5 hour prior to endothelial permeability measurement. Following blood withdrawing, organ vasculature was flushed free of blood by gentle infusion of 10 ml prewarmed PBS through left ventricle. Lungs were excised, and weighed before and after being dried at 70°C for 16 hours. Dry tissues were homogenized in formamide, incubated at 60°C for 16 hours, centrifuged, and supernatant absorbance at 620 and 740 nm was recorded. Tissue heme pigment contamination was corrected using $A_{740}$ readings. Tissue EBD content (ng/mg dry tissue) was calculated by comparing tissue supernatant $A_{620}$ readings to an EBD standard curve.

To examine the effect of targeted inhibition of REC or BMDEPC proliferation on endothelial barrier recovery, WT and chimeric mice were injected with saline or LPS, and then with Dox (0.5 mg/mouse, i.p.) 36 hours later. Endothelial permeability was measured 48 hours after LPS injection.

**BrdU labeling of proliferating cells in vivo:**

The *in situ* cell proliferation kit (Roche Diagnostics) was used for the detection of proliferating RECs or BMDEPCs. To label proliferating cells *in vivo*, mice were injected with bromodeoxyuridine (BrdU, 100 μg/kg, i.v.) at 44 hours after LPS injection. BrdU+/rtTA+ RECs or BrdU+/GFP+ BMDEPCs were detected by immunofluorescence staining of lung sections at 48 hours post-LPS, or quantified using FACS analysis at 8 weeks after LPS injection.

**Immunofluorescence staining:**

Following perfusion of animals with 10 mL10% neutral buffered formalin via left ventricle puncture, lungs were removed, prepared and embedded in OCT compound. Cryosections (10 μm) were prepared, briefly fixed with acetone, and stained overnight at 4°C with rat anti-CD31 (BD Bioscience, San Jose, CA), rat anti-Ve-cadherin (eBioscience, San Diego, CA) and rabbit anti-aquaporin 5 (Abcam, Cambridge, MA) antibodies, followed by washing and staining with Alexa Fluor 594 anti-rat or Alexa Fluor 647 anti-rabbit secondary antibody for 2 hours (Invitrogen, Carlsbad, CA). The slides were fixed for 20 minutes with 4% paraformaldehyde,
permeabilized in 0.1% Triton-X at RT for 5 minutes (for GFP staining) or treated with 2M hydrogen chloride containing 0.3% Triton-X at 37° for 20 minutes (for BrdU staining). After blocking in 5% serum and 0.25% bovine serum albumin for 1 hour, sections were stained with biotin-anti-BrdU, rabbit anti-rtTA or goat anti-GFP antibody (all from Abcam, Cambridge, MA) overnight at 4°C, washed, and subsequently stained with Alexa Fluor 594 anti-rabbit or Alexa Fluor 488 anti-goat secondary antibody, or Alexa Fluor 488 Streptavidin (all from Invitrogen, Carlsbad, CA) at 4°C for 2 hours. The slides were washed, nuclear counterstained with TO-PRO-3 dye (Life Technologies, Norwalk, CT) and mounted with Vectashield antifading medium (Vector Laboratories, Burlingame, CA). Z stacks of optical sections were captured on FluoView FV300 Confocal Laser Scanning Microscope (Olympus, Melville, NY) using 100x oil immersion objective lenses. Images were analyzed and 3D image reconstructed using Image J software (http://imagej.nih.gov/ij/).

For studying time course of REC or BMDEPC proliferation, or BMDEPC recruitment, cryosections (3 μm) of lungs were prepared from control mice and mice 12, 24, 48 or 96 hours after LPS injection, and stained with BrdU plus rtTA or BrdU plus GFP antibodies, or GFP antibody alone, followed by Alexa Fluor 594 or Alexa Fluor 488 conjugated secondary antibodies, and nuclei counterstained with 4',6-diamidino-2-phenylindole (DAPI). Numbers of rtTA+/BrdU+ proliferating RECs, GFP+/BrdU+ proliferating BMDEPCs or GFP+ BMDEPCs were identified, counted and expressed as a percentage of total cells as revealed by DAPI nuclear staining.

To compare the relative contribution of RECs versus BMDEPCs to endothelial barrier repair, adjacent sections from the same lung of mice 48 hours after saline or LPS injection were stained with rtTA plus BrdU or GFP plus BrdU antibodies, and nuclei counterstained with DAPI. Numbers of rtTA+/BrdU+ proliferating RECs and GFP+/BrdU+ proliferating BMDEPCs were counted, expressed as a percentage of total cell nuclei and compared.
To examine the effect of targeted inhibition of REC or BMDEPC intrinsic NF-κB activity on RECs or BMDEPCs proliferation, lung cryosections (3 μm) were prepared from the 4 chimeric mouse strains 48 hours after LPS or saline injection, and stained with rtTA plus BrdU antibodies. Numbers of proliferating RECs or proliferating BMDEPCs and total BrdU+ proliferating cells were counted. Proliferating RECs or BMDEPCs were expressed as a percentage of total BrdU+ proliferating cells and compared.

Fluorescence activated cell sorting (FACS):

To quantify REC- and BMDEPC-derived new ECs, and engrafted BMDEPC, two groups of EC-rtTA-GFP-BM mice were injected with saline or LPS (5 mg/kg, i.p.) and then with BrdU (100 μg/kg, i.v. at 44 hours after LPS injection). At 8 weeks after saline or LPS injection, lungs were harvested, finely minced and digested with collagenase A (1 mg/ml, Worthington, NJ) at 37°C for 60 minutes. The resulting digest was filtered through a 40-μm cell strainer. The single cell suspension was treated with red blood cell lysis buffer to remove red blood cells, washed and stained with PE-anti-mouse CD45 and PE/Cy7-anti-mouse CD31 antibodies (BD Biosciences, San Jose, CA). After fixation and permeabilization, half of the cells were treated with DNase I (BD Biosciences, San Jose, CA) and stained with PerCP/Cy5.5-anti-BrdU (BD Biosciences, San Jose, CA), Alexa Fluor 488-anti-GFP (Life Technologies, Norwalk, CT) and anti-rtTA (Abcam, Cambridge, MA) antibodies. The other half of the cells was stained with Alexa Fluor 488-anti-GFP and anti-eNOS antibodies (Santa Cruz Biotech, Dallas, TX). Primary antibody staining was followed by Alexa Fluor 647-conjugated secondary antibody (Invitrogen, Carlsbad, CA), where anti-rtTA or anti-eNOS antibodies were used.

To evaluate BM chimerism, BM or peripheral blood mononuclear cells were isolated from donors, recipients and chimeras using Ficoll density gradient centrifugation, and stained with PE/Cy7-anti-CD31 plus Alexa Fluor 488-anti-GFP or PE/Cy7-anti-CD31 plus anti-rtTA antibodies, followed by Alexa Fluor 647-conjugated secondary antibody. Fluorescence minus one (FMO) controls were stained in parallel in all analyses.
FACS acquisition and sorting were conducted using BD FACSVerse flow cytometer (BD Biosciences, San Jose, CA) at the Feinstein Institute for Medical Research flow cytometry core facility. Raw data were processed with FlowJo software (Treestar, Ashland, OR). Forward/side scatter properties were used to exclude cell debris and fragments. ECs were identified and gated within as CD45-/CD31+ cells. The EC population was further analyzed to quantify REC- or BMDEPC-derived new ECs, defined as CD45-/CD31+/BrdU+/rtTA+ cells or CD45-/CD31+/BrdU+/GFP+ cells. To quantify engrafted BMDEPCs, ECs were identified and gated within as CD45-/CD31+/eNOS+ cells, and GFP+ cells within the CD45-/CD31+/eNOS+ cell population were identified and quantified. To phenotype GFP+ cells, GFP+ cell population was sorted from lung single cell suspension of Tie2-GFP mice at 48 hours after LPS injection, and analyzed for their expression of hematopoietic cell marker, CD45, and EC markers, CD31 and eNOS.

*Western blot:*

Membrane and cytoplasmic proteins were extracted as we have previously described. In brief, lungs were homogenized in extracting buffer containing no detergent, and centrifuged at 12,000 g for 15 minutes. The resulting pellet and supernatant were collected as membrane preparation and cytoplasmic protein, respectively. The membrane pellets were resuspended and incubated in the same buffer containing 0.1% Triton X-100 at 4°C for 2 hours, homogenized and centrifuged at 12,000 g for 30 minutes. The second supernatant was collected as membrane protein.

Equal amount of proteins (20 μg/lane) were separated on 7.5% or 4–20% (w/v) gradient polyacrylamide gels (BioRad, Hercules, CA) under denaturing conditions. Proteins were electroblotted to PVDF membrane (EMD Millipore, Billerica, MA). After incubation in blocking solution (5% dry milk in TBST) at room temperature for 2 hours, the membrane was incubated with primary antibodies against phospho-MLC2 (Ser19), myosin light chain (MLC)2 (Cell Signaling, Danvers, MA), VE-cadherin (Santa Cruz Biotech, Dallas, TX), vascular cell adhesion
molecule (VCAM)-1 (Santa Cruz Biotech, Dallas, TX) or actin at room temperature for 1 hour. The membrane was washed and incubated with secondary antibody conjugated to horseradish peroxidase at room temperature for 1 hour. Peroxidase labeling was detected using SuperSignal West Pico Kit (Thermo Fisher Scientific, Waltham, MA). The same membrane for p-MLC2 blotting was re-blotted to MLC2 antibody after strapping off the p-MLC2 antibody, and was subsequently re-blotted to actin antibody without stripping. The western blot p-MLC2, MLC2 and VE-cadherin bands were quantified using densitometry, and expressed as $10^4$ pixels.

Measurement of tissue levels of cytokines:

Tissue levels of TNF-α, IL-1β, IL-6, chemokine (C-X-C motif) ligand 1 (CXCL1) were measured using ELISA kits (eBioscience, San Diego, CA; R & D, Minneapolis, MN).

Statistical Analysis:

Data were expressed as mean ± SEM and analyzed using ANOVA or Kruskal-Wallis rank test followed by Holm-Sidak method or Student-Newman-Keuls Method for post hoc analysis. The null hypothesis was rejected at 5% level.

Supplemental References:

**Supplemental Materials**

**Supplemental Table I. Transgenic Mice Used in This Study**

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<thead>
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<th>Strains</th>
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<td>EC-rtTA</td>
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<td>EC-I-κBαmt</td>
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**Supplemental Table II. Chimeric Mice Used in This Study**

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<th>Donors</th>
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<td>EC-rtTA-GFP-BM</td>
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Supplemental Figures

Supplemental Figure I

A  Tie2-GFP (donor)  EC-rTA-GFP-BM (chimera)  EC-rTA (recipient)

B  EC-I-κBαmt (donor)  WT-EC-I-κBα-BM (chimera)  WT (recipient)

C  WT (donor)  EC-I-κBα-WT-BM (chimera)  EC-I-κBαmt (recipient)
Supplemental Figure I. Fluorescence activated cell sorting (FACS) analysis of BMMNCs demonstrates high level of BM chimerism.

Two months after BM transplant, BM mononuclear cells (BMMNCs) were isolated from donors, recipients and chimeras, stained with CD31 plus GFP or CD31 plus rtTA antibodies, and subjected to FACS analysis. The degree of BM chimerism was evaluated based on the percentage of donor-derived CD31+/GFP+ or CD31+/rtTA+ endothelial progenitor cells (EPCs) in BMMNC population of the chimera.

A. Representative FACS pictures show percentage of CD31+/GFP+ EPCs (upper right quadrant) in BMMNCs from Tie2-GFP (donor), EC-rtTA-GFP-BM (chimera) and EC-rtTA (recipient) mice, demonstrating high degree of BM chimerism.

B. Representative FACS pictures show percentage of CD31+/rtTA+ EPCs (upper right quadrant) in BMMNCs of EC-IκBαmt (donor), WT-EC-IκBα-BM (chimera) and WT (recipient) mice, demonstrating high degree of BM chimerism.

C. Representative FACS pictures show percentage of CD31+/rtTA+ EPCs (upper right quadrant) in BMMNCs of WT (donor), EC-IκBα-WT-BM (chimera), and EC-IκBαmt (recipient) mice, demonstrating high degree of donor BM chimerism.
Supplemental Figure II. Phenotypic characterization of GFP+ cells in Tie2-GFP mouse lungs.

FACS analysis of lung cells from Tie2-GFP donor mice 48 hours after LPS injection shows that 99% of the GFP+ cells are CD45−/CD31+/eNOS+, confirming their endothelial phenotype.
Supplemental Figure III

Supplemental Figure III. PCR photograph shows that Dox treatment of EC-\(\kappa B\alpha\text{-WT-BM}\) mice induces high level of \(\kappa B\alpha\text{mt}\) mRNA expression. EC-\(\kappa B\alpha\text{-WT-BM}\) mice were injected with saline or doxycycline (Dox, 0.5 mg/mouse, i.p.) 36 hours after LPS injection. Lung tissue level of \(\kappa B\alpha\text{mt}\) mRNA was determined 48 hours after LPS and 12 hours after Dox injection. Tissue level of GAPDH mRNA was also determined as an internal control.
Supplemental Figure IV

A

TNF-α, (pg/mg protein)

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B

IL-1β, (pg/mg protein)

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C

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D

CXCL1, (ng/mg protein)

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E

p-MLC

MLC

Actin

F

VE-cad-M

Actin

VE-cad-C

Actin

Supplemental Figure IV
**Supplemental Figure IV.** EC-restricted inhibition of NF-κB activity at 48 hours had no effect on lung tissue levels of cytokines and markers of inter-endothelial junction disruption.

A to D, WT and EC-IκBαmt (I-κB) mice were injected with saline or LPS. Lungs were harvested 6 or 48 hours after LPS and 12 hours after Dox injection. Tissue levels of TNF-α (A), IL-1β (B), IL-6 (C) and chemokine ligand 1 (CXCL1, D) were measured. Mean ± SEM of 5 mice per group. *, p < 0.05, compared to control groups at each time point.

E and F, Membrane and cytoplasmic proteins were extracted from lungs 3, 6 or 48 hours after LPS and 12 hours after Dox injection. Equal amount of membrane or cytoplasmic protein (20 µg/lane) was separated on SDS-PAGE. The amount of phosphorylated myosin light chain 2 (E, p-MLC), MLC2 (E, MLC2), membrane-bound VE-cadherin (F, VE-cad-M) or cytoplasmic VE-cadherin (F, VE-cad-C) protein was detected. The same membrane was first blotted to p-MLC2 antibody, re-blotted to MLC2 antibody after strapping off the p-MLC2 antibody, and was subsequently re-blotted to actin antibody without stripping. The Western blot bands were quantified using densitometry, analyzed and summarized in Supplemental Figure V. W-C, WT control, I-C, EC-IκBαmt control, W-3, WT 3 hours, I-3, EC-IκBαmt 3 hours, W-6, WT 6 hours, I-6, EC-IκBαmt 6 hours, W-48, WT 48 hours, I-48, EC-IκBαmt 48 hours.
**Supplemental Figure V.** LPS and NF-κB blockade had no effect on lung tissue levels of markers of inter-endothelial junction disruption at 48 hours

WT and EC-I-κBαmt (I-κB) mice were injected with saline or LPS. Lungs were harvested 3, 6 or 48 hours after LPS and 12 hours after Dox injection. Membrane and cytoplasmic proteins were extracted, and tissue levels of phosphorylated myosin light chain 2 (p-MLC, A) and MLC2 (B) or membrane-bound VE-cadherin (VE-Cad-M, C) proteins were detected using Western blot. The Western blot protein bands were quantified using densitometry, and expressed as $10^4$ pixels. Mean ± SEM of 4 independent experiments. *, p < 0.05, compared to control group. #, p < 0.05, compared to WT-LPS group at each time point.