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Bone Marrow-Derived Progenitor Cells Are Important for Lung Repair after Lipopolysaccharide-Induced Lung Injury

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Tissue repair often occurs in organs damaged by an inflammatory response. Inflammatory stimuli induce a rapid and massive release of inflammatory cells including neutrophils from the bone marrow. Recently, many studies suggested that bone marrow cells have the potential to differentiate into a variety of cell types. However, whether inflammatory stimuli induce release of bone marrow-derived progenitor cells (BMPCs), or how much impact the suppression of BMPCs has on the injured organ is not clear. Here we show that LPS, a component of Gram-negative bacterial cell walls, in the lung airways, induces a rapid and massive mobilization of BMPCs into the circulation in mice. BMPCs accumulate within the inflammatory site and differentiate to become endothelial and epithelial cells. Moreover, the suppression of BMPCs by sublethal irradiation before intrapulmonary LPS leads to disruption of tissue structure and emphysema-like changes. Reconstitution of the bone marrow prevents these changes. These data suggest that BMPCs are important and required for lung repair after LPS-induced lung injury. The Journal of Immunology, 2004, 172: 1266–1272.

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A n increased peripheral white cell count (leukocytosis) is a clinical symptom suggesting the presence of bacterial infection or inflammation. Inflammatory stimuli, such as a microbes or LPS, induce a rapid and massive release of neutrophils from the bone marrow (1). Later, monocytes, lymphocytes, and platelets are also released. Released inflammatory cells are important for host defense and resolution of an inflammatory response. They migrate into the inflammatory site, release inflammatory mediators (e.g., reactive oxygen species, cytokines, proteases), and kill bacteria, but also cause tissue disruption and injury. The bone marrow is the main source of the inflammatory cells.

Recently, bone marrow is becoming a recognized source of progenitor cells for several cell types (2), including endothelial cells (3), epithelial cells (4–6), myocytes (7, 8), and neurons (9, 10). Bone marrow-derived endothelial progenitor cells can circulate in the peripheral blood and track to other organs (3, 11). Jiang et al. established a multipotent adult progenitor cell line from bone marrow cells and demonstrated its capacity to differentiate into cells of many types (12).

At inflammatory sites, tissue damage and repair often occur. The initial injury and acute inflammatory response can result in apoptosis and necrosis of parenchymal cells, including endothelial and epithelial cells (13). The repair process may require replacement of these dead cells that are not able to divide with bone marrow-derived progenitor cells (BMPCs) that are capable of differentiating in endothelial or epithelial directions. Thus, our studies tested the hypothesis that inflammatory stimuli induce release of progenitor cells from bone marrow, and these cells are important for repairing the tissue damage induced by inflammatory mediators.

Materials and Methods

Lethal irradiation and reconstitution of bone marrow

Mice transgenic for enhanced green fluorescent protein (GFP) on a background of a C57BL/6 strain were established at the Research Institute for Microbial Diseases (Osaka University, Japan) (14). Adoptive transfer of fetal liver cells was performed according to a previously described method (15). Briefly, embryos were generated from crosses of GFP mice. The expression of GFP in embryos was examined by the naked eye using UV excitation light. Fetal livers were harvested from day 13.5 GFP embryos (where day 0.5 is defined as the morning the plugs are identified) and placed in BMC medium (HBSS, 10 nM HEPES, 2.5% FBS). Single-cell suspensions were prepared by passage through a 26-gauge needle. Recipient C57BL/6 male mice were irradiated using doses of 8 Gy and 4 Gy, separated by 3 h. Their bone marrow was reconstituted by injecting fetal liver cells (2 × 10^6 cells/200 μl medium) i.v. through the tail vein. The mice were then maintained under sterile conditions. All experiments were performed in accordance with Animal Studies Committee regulations at Tohoku University School of Medicine.

LPS-induced lung injury

LPS from Escherichia coli serotype 055:B5 was obtained from Sigma-Aldrich (St. Louis, MO). Induction of lung injury was performed as previously described (16). Briefly, recipient mice were anesthetized by ketamine hydrochloride. While anesthetized, an intranasal insufflation was conducted by inhalation of LPS (20 μg/mouse in PBS) placed on one nostril. Control mice received only PBS.

Histological analysis of recipient lungs

Lungs were fixed for 5 min by instillation of 4% paraformaldehyde-PBS through a tracheal catheter at a transpulmonary pressure of 15 cmH₂O. After tracheal ligation, harvested lungs were fixed in 4% paraformaldehyde-PBS overnight. Fixed lungs were embedded in paraffin and sectioned by standard methods. The same fixed lungs were washed in sucrose-PBS.

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embedded in Tissue-Tek OCT compound (Miles Scientific, Naperville, IL), and frozen in liquid nitrogen for cryosectioning. The paraffin sections were processed for staining with H&E or for immunohistochemistry. GFP was detected with a rabbit polyclonal anti-GFP Ab (Abcam, Cambridge, U.K.). HRP-labeled goat anti-rabbit IgG (Vector, Burlingame, CA) was used as a secondary Ab. Samples were subsequently incubated with avidin-biotin HRP complex (ABC Elite; Vector). The final reaction product was visualized with 3-3’-diaminobenzidine (Sigma-Aldrich). Sections were counterstained with hematoxylin. Cryosections were processed for immunofluorescence staining. Alveolar epithelial cells were identified using a murine monoclonal anti-cytokeratin 5/6 (Chemicon, Temecula, CA) and a goat Alexa 488-conjugated anti-mouse IgG Ab (Molecular Probes, Eugene, OR). Endothelial cells were identified using a rat biotin-conjugated monoclonal anti-murine CD34 Ab (BD PharMingen, San Diego, CA) and 7-amino-4-methylcoumarin-3-acetic acid-conjugated streptavidin (Immuno-technic, Marseille, France). Hemopoeitic cells were identified using a rat PE-conjugated anti-murine CD45 Ab (BD PharMingen).

**Cell sorting and flow cytometry**

A total of 0.5–1.0 ml of blood was obtained from each mouse. RBC were depleted by RBC lysis buffer (0.15M NH4Cl, 0.01M KHCO3, 0.1 mM EDTA-2Na, pH 7.2). Stem cell Ag (Sca)-1/H11001

The sections were then incubated with the FITC-conjugated goat anti-endothelial cell adhesion molecule-1 (PECAM-1) mAb (BD PharMingen). C with the rat at room temperature for 1 h, and incubated overnight at 4 °C, rinsed in PBS. The cells were preincubated in 5% normal goat serum-PBS and then fixed in 100% methanol for 10 min at −20°C, permeabilized in 0.1% Triton X-100 for 20 min, and rinsed in PBS. The cells were preincubated in 5% normal goat serum-PBS at room temperature for 1 h, and incubated overnight at 4°C with the rat anti-mouse Flk-1 mAb (BD PharMingen) and the rat anti-mouse platelet endothelial cell adhesion molecule-1 (PECAM-1) mAb (BD PharMingen). The sections were then incubated with the FITC-conjugated goat anti-mouse IgG Ab (1:100; BD PharMingen) for 1 h at room temperature. Coverslips were mounted in PermaFluor (Shandon, Pittsburgh, PA). They were analyzed by fluorescent microscopy (Leica, Solms, Germany).

**Bone marrow suppression and reconstitution with bone marrow cells**

Recipient 8-wk-old male C57BL/6 mice were irradiated with 5 Gy to suppress the bone marrow (20). Immediately after the irradiation, LPS-induced lung injury was initiated as previously described. Bone marrow cells were obtained from pelvic, femoral, and peroneal bones of GFP mouse donors, and 1 × 107 mononuclear cells plated on four-well culture slides coated with human fibronectin (Biocoat; Becton Dickinson Labware, Bedford, MA). Growth medium was changed every 3 days. After 7 days of culture, nonadherent cells were removed by a thorough washing with PBS, and adherent cells underwent cytological analysis. Cells were fixed in 100% methanol for 10 min at −20°C, permeabilized in 0.1% Triton X-100 for 20 min, and rinsed in PBS. The cells were preincubated in 5% normal goat serum-PBS at room temperature for 1 h, and incubated overnight at 4°C with the rat anti-mouse Flk-1 mAb (BD PharMingen) and the rat anti-mouse platelet endothelial cell adhesion molecule-1 (PECAM-1) mAb (BD PharMingen). The sections were then incubated with the FITC-conjugated goat anti-mouse IgG Ab (1:100; BD PharMingen) for 1 h at room temperature. Coverslips were mounted in PermaFluor (Shandon, Pittsburgh, PA). They were analyzed by fluorescent microscopy (Leica, Solms, Germany).

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morphologically consistent with alveolar epithelial cells or endothelial cells, appeared in the parenchyma of LPS-treated recipient lungs (Fig. 1B), but not in PBS-treated control lungs (Fig. 1A). These cells were not present before or at 4 h after LPS, and only a very few GFP-positive cells appeared 24 h after LPS-treatment and were numerous at 1 wk (Fig. 1C). To confirm the cell type of the GFP-positive cells in LPS-treated recipient mice, triple-color immunofluorescent staining was performed using CD45 to mark hemopoietic cells and either cytokeratin to mark epithelial cells (22) or CD34 to mark endothelial cells (23). Flat GFP<sup>+</sup> cytokeratin<sup>-</sup>CD45<sup>-</sup> cells were present in the alveolar walls of LPS-treated recipient lungs, but not in control lungs (Fig. 2, A and B), suggesting that BMPC had differentiated to an alveolar epithelial phenotype. Similarly, flat GFP<sup>+</sup>CD34<sup>-</sup>CD45<sup>-</sup> cells were also observed, suggestive of differentiation toward pulmonary capillary endothelial cells (Fig. 2, C and D). Although CD45<sup>-</sup> cells were identified in the same lung sections, no cell stained simultaneously for both CD45 and cytokeratin, or for CD45 and CD34.

**LPS induces a rapid mobilization of BMPC into the circulation, and BMPCs are thought to exist mainly in bone marrow**

To participate in repairing damaged lung tissues, BMPCs would emigrate from bone marrow to damaged tissues via the vascular system. Therefore, whether LPS in the lung airways increased the number of BMPCs in peripheral blood was determined. We focused on EPCs because they are present in peripheral blood and can differentiate mature endothelial cells (2–4). Bone marrow-derived circulating EPCs express markers of both hemopoietic stem cells and hemangioblasts, such as Sca-1 and Flk-1/VEGF-R2. C57BL/6 male mice were given LPS or PBS intranasally. At 4 h after administration, mice were killed and peripheral blood was
collected. The proportion of Sca-1-positive (Sca-1⁺) cells in PBMCs was not different in LPS-treated and control mice. These Sca-1⁺ cells were collected from PBMCs using magnetic sorting techniques, and Sca-1⁺ cells were stained with both anti-Sca-1 and anti-Flk-1 Abs. Flow cytometry demonstrated that Sca-1⁺Flk-1⁺ cells were significantly increased in peripheral blood of LPS-treated mice (Fig. 3, A and B). To further evaluate EPC mobilization, we performed a murine EPC culture assay (17–19). PBMCs were isolated from peripheral blood of mice treated with PBS or LPS intranasally at 4 h after administration. After 1 wk of culture, the number of EPCs confirmed by a combination of Flk-1 (Fig. 4, C and D) and PECAM-1 expression (data not shown) documented increased circulating EPCs in the peripheral blood of LPS-treated mice vs control mice (275 ± 10 vs 52 ± 3 cells/mm²; *p < 0.01) (Fig. 4, A–F).

**BMPCs are required for normal repair of lungs damaged by inflammation induced by LPS**

Our data suggest that BMPCs rapidly emigrate from the bone marrow to the circulation in response to LPS administration within lungs and migrate into the inflammatory site, then differentiate to form endothelial and epithelial cells. However, whether BMPCs are critical for repairing the damaged lung parenchyma induced by inflammatory stimuli still remains an important question. To address this question, mice were irradiated to damage the BMPCs. C57BL/6 male mice were irradiated with 5 Gy to induce bone marrow suppression (20), but not lethal irradiation. Irradiated mice and control mice were treated with either LPS or PBS by nasal insufflation immediately after irradiation. The number of neutrophils in bronchoalveolar lavage and lung histology were not different between irradiated and control mice at 4 h after LPS, suggesting that the degree of the LPS-induced lung injury was the same. One week after the administration of LPS or PBS, the lungs were fixed by intratracheal instillation of paraformaldehyde at a constant airway pressure and evaluated morphometrically. No abnormalities were observed in lungs following irradiation or LPS alone, compared with uninjured lungs (Fig. 5, A, D, and E). However, surprisingly, irradiated mice given LPS developed emphysema-like lesions (Fig. 5B). The Lm was determined to quantitate the degree of alveolar enlargement. The Lm of the group treated with both irradiation and LPS was significantly larger than that of the group treated with either irradiation or LPS alone (Fig. 6; 97 ± 9 μm vs 62 ± 3 μm, 67 ± 2 μm, *p < 0.01).

To test the hypothesis that BMPCs are required to repair the injured lung tissues, mice were irradiated with a nonlethal dose (5 Gy), given LPS by nasal insufflation, and immediately reconstituted with GFP-expressing bone marrow cells or with medium alone. Mice reconstituted with hemopoietic stem cells did not develop the emphysema-like lesions 1 wk after instillation of LPS, compared with mice given medium alone (Fig. 5C and Fig. 6; Lm 73 ± 3 μm vs 92 ± 6 μm (data not shown), *p < 0.01). Thin flat GFP-positive cells appeared in the parenchyma of lungs from mice that were irradiated, challenged with LPS, and reconstituted with GFP-positive bone marrow cells (Fig. 7A and Fig. 8). Moreover, GFP⁺ cytokeratin⁺ CD45⁻ alveolar epithelial cells and GFP⁺CD34⁻CD45⁻ endothelial cells were present in the parenchyma of LPS-treated and irradiated recipient murine lungs of reconstituted mice (Fig. 7, B and C).

**Discussion**

In this study, we examined whether BMPCs were released to the bloodstream by inflammatory stimuli and migrated to an inflammatory site. For this purpose, the distribution of BMPCs in the lungs was evaluated following acute pulmonary inflammation. The C57BL/6 mice were reconstituted with fetal liver cells from GFP-transgenic murine embryos to generate mice with GFP-expressing bone marrow cells. The distribution of bone marrow-derived cells was analyzed by tracing GFP-positive cells. We found that LPS administration into the airspace increased the number of GFP-positive cells in alveoli after 1 wk. Because GFP-positive cells were...
not present in the alveolar wall in PBS-administered lungs, inflammation was required for BMPCs to be released from bone marrow and to sequester and migrate to alveoli.

Inflammatory stimuli, such as LPS, are known to induce a rapid and massive release of inflammatory cells from bone marrow (1). We showed that the number of cells expressing Sca-1 and Flk-1, markers of bone marrow-derived circulating EPCs, also increased in peripheral blood after LPS administration (Fig. 3, A and B). EPCs exist in a fraction of peripheral mononuclear cells (3, 17–19). Therefore, we cultured peripheral mononuclear cells obtained from LPS-treated mice and found that adherent cells, whose phenotype was compatible with endothelial cells (3, 17–19), increased in the LPS group (Fig. 4, A–F). This confirms the idea that the circulating progenitor cells increase after LPS administration. These data indicate that the signals produced by inflammatory stimuli promote the release of EPCs, and presumably all BMPCs. Then, LPS induces a rapid release of not only inflammatory cells but also BMPCs from bone marrow to the circulation. BMPCs existing in the alveolar walls were shaped thin and flat (Fig. 1B), and stained for cytokeratin as a marker of epithelial cell or CD34 as a marker of endothelial cells. LPS administration induces severe damage to lung parenchymal cells, resulting in apoptosis and necrosis (13). Because these dead cells are not able to divide, other cells must replace them to repair the tissue and keep the organ homeostasis. BMPCs are capable of differentiating to several phenotypes (2–

![FIGURE 5](http://www.jimmunol.org/)

LPS induces emphysema-like changes in mice when BMPCs were suppressed by irradiation. A–E, Lung H&E-stained sections of mice 1 wk following: A, intranasal PBS; B, intranasal LPS in irradiated (5 Gy) mice; C, intranasal LPS in irradiated and reconstituted mice; D, intranasal PBS in irradiated (5 Gy) mice; and E, intranasal LPS. Scale bars, 50 μm.

![FIGURE 6](http://www.jimmunol.org/)

Changes in the Lm as the morphometric parameter for pulmonary emphysema. Results were obtained from five mice in each group. Values are mean ± SEM. *, p < 0.01 vs LPS alone; †, p < 0.01 vs irradiation alone; ‡, p < 0.01 vs mice reconstituted with GFP-expressing bone marrow cells.

![FIGURE 7](http://www.jimmunol.org/)

GFP-positive endothelial cells and epithelial cells in lungs of irradiated and reconstituted mice given LPS. A, Paraffin sections were immunostained for GFP (brown). B and C, Immunostaining for cytokeratin and CD45 (B) or CD34 and CD45 (C). Color staining: GFP, green; cytokeratin or CD34, blue; CD45, red. Note GFP+/cytokeratin+/CD45− and GFP+/CD34+/CD45− cells were observed in the alveolar walls (light blue, arrowheads). Scale bars, 25 μm.
Our findings demonstrate the possibility of the involvement of BMPCs in repairing damaged tissue.

To investigate the importance of BMPCs in damaged tissue, we examined LPS-induced lung injury in mice whose bone marrow was suppressed. Although no structural change was present in the lungs at 1 wk after LPS administration in normal mice whose bone marrow was intact, LPS induced emphysema-like changes in bone marrow-suppressed mice. In normal mice, thin and flat-shaped BMPCs were incorporated into the structure of the alveolar walls after LPS administration (Fig. 1B). Therefore, the absence of these differentiated BMPCs might cause the failure to repair damaged alveoli in bone marrow-suppressed mice, resulting in emphysema-like changes in the lung parenchyma. To test this idea, we performed GFP-positive bone marrow transplantation to bone marrow-suppressed mice and induced a lung injury. Bone marrow transplantation inhibited the development of emphysema-like lesions in the lungs treated with both irradiation and LPS. In these lungs, GFP+ cytokeratin CD45+ epithelial cells and GFP+ CD34+ CD45+ endothelial cells were present in the alveolar walls. These data strongly suggest that BMPCs are required for repairing damaged epithelium and endothelium induced by LPS-induced inflammation in the lung.

Mesenchymal cells play an important role in tissue repair during wound healing. In contrast, inappropriate proliferation of fibroblasts causes tissue fibrosis, including pulmonary fibrosis. Recent studies reported that bone marrow-derived circulating fibroblasts contributed to tissue repair during wound healing and to the genesis of subepithelial fibrosis in asthma (24, 25). Our studies did not address whether bone marrow-derived cells differentiate toward lung mesenchymal fibroblasts as well as endothelial and epithelial cells in LPS-induced lung injury, but recruitment and differentiation of mesenchymal cells derived from the bone marrow may be responsible for proper repair following lung injury. Further studies, including primary cultures of mesenchymal fibroblasts and alveolar epithelial cells from GFP chimeric mice, will reveal the roles of bone marrow-derived cells in lung repair and lung diseases such as pulmonary fibrosis.

Recently, several studies reported that bone marrow cells adopt the phenotype of other kinds of cells by spontaneous cell fusion, not by differentiation (26–28). In our study, whether GFP+ cytokeratin CD45+ alveolar epithelial cells and GFP+ CD34+ CD45+ endothelial cells were generated by differentiation of BMPCs or fusion of BMPCs with existing parenchymal cells still remains a question. However, our data strongly suggest that BMPCs are indispensable for complete repair of damaged lung tissue regardless of whether BMPCs differentiate or fuse.

These findings show that inflammatory stimuli present in bacterial pneumonia induce a rapid release of not only inflammatory cells but also BMPCs from bone marrow to the circulation. BMPCs sequester and migrate into the inflammatory site, and then differentiate to become endothelial and epithelial cells. BMPCs can differentiate toward several phenotypes (2–10), and they are important for lung repair after LPS-induced lung injury. Because inflammatory stimuli induce the emigration of progenitor cells from bone marrow to the inflammatory site, gene therapy using BMPCs (29) may facilitate resolution and repair of inflammatory lesions. Moreover, induction of BMPCs may be useful in repairing lung parenchyma of patients suffering from diseases where lung destruction is an important component, such as emphysema. The results from our study indicate that BMPCs may have utility for therapeutic use in lung disease.

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