

# The Critical Role of Hematopoietic Cells in Lipopolysaccharide-induced Airway Inflammation

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**Rapid and selective recruitment of neutrophils into the airspace in response to LPS facilitates the clearance of bacterial pathogens. However, neutrophil infiltration can also participate in the development and progression of environmental airway disease. Previous data have revealed that Toll-like receptor 4 (tlr4) is required for neutrophil recruitment to the lung after either inhaled or systemically administered LPS from *Escherichia coli*. Although many cell types express tlr4, endothelial cell expression of tlr4 is specifically required to sequester neutrophils in the lung in response to systemic endotoxin. To identify the cell types requiring tlr4 expression for neutrophil recruitment after inhaled LPS, we generated chimeric mice separately expressing tlr4 on either hematopoietic cells or on structural lung cells. Neutrophil recruitment into the airspace was completely restored in tlr4-deficient mice receiving wild-type bone marrow. By contrast, wild-type animals receiving tlr4-deficient marrow had dramatically reduced neutrophil recruitment. Moreover, adoptive transfer of wild-type alveolar macrophages also restored the ability of tlr4-deficient recipient mice to recruit neutrophils to the lung. These data demonstrate the critical role of hematopoietic cells and alveolar macrophages in initiating LPS-induced neutrophil recruitment from the vascular space to the airspace.**

**Keywords:** innate immunity; lipopolysaccharide; macrophage; neutrophil; toll-like receptor

Neutrophil recruitment to the lung represents an important first line of defense against bacterial infections. However, the products of these cells can be toxic to the lung and their recruitment must be carefully regulated to maintain homeostasis. Neutrophilic inflammation is seen after pulmonary exposure to bacteria, bacterial products, or inhaled environmental toxins and can be associated with severe airway disease (1–7). Inhaled endotoxin, or LPS, from gram-negative bacteria causes neutrophilic inflammation and decrements in pulmonary function (8), and is associated with exacerbations of allergic asthma (9–12). Chronic inhalation of LPS can cause chronic airway remodeling that is dependent on the recruitment of neutrophils from the vascular space to the airspace (13). Although neutrophil recruitment into the airways has evolved as an effective host response to combat bacterial infections, dysregulation of this innate inflammatory

response can lead to undesirable tissue injury, including acute lung injury and adult respiratory distress syndrome (14). Despite its clinical importance, the regulation of neutrophil migration remains poorly understood. Understanding the mechanisms that orchestrate neutrophil recruitment into the lung in response to specific environmental toxins and microorganisms might allow us to develop novel therapies to combat pathogens while minimizing tissue damage.

Toll-like receptor 4 (tlr4) is a membrane-associated molecule critical for LPS-induced cell activation (15–19). Many cell types within the lung express tlr4 and respond to stimulation by LPS (20). Hematopoietic-derived tlr4-expressing cells in the lung include macrophages (18), neutrophils (21), lymphocytes (22), and dendritic cells (23). In addition, non-hematopoietic-derived cells, such as airway epithelia (24–26) and endothelia (27), express tlr4 and respond to stimulation by LPS. Recent work by Andonegui and coworkers (27) suggested that tlr4 expression on endothelial cells is sufficient to sequester neutrophils into the lung after a systemic LPS challenge, but it is not known whether the response to inhaled LPS requires expression of tlr4 on endothelia or a combination of other cell types. Skerrett and colleagues (28) demonstrated that airway epithelia are critical in the response to inhaled LPS. They demonstrated attenuation of lung inflammation by disruption of nuclear factor- $\kappa$ B translocation in airway epithelia. Previously, a study by Koay and coworkers suggested that macrophages might be involved in this process. In that study, macrophage depletion with clodronate abrogates the inflammatory response to intratracheally administered LPS (29, 30). Unfortunately, many cell types can be affected by clodronate, including endothelial cells (31), so the relative role of lung macrophages remains unclear. This compound also has additional effects in the lung, including the following: inhibition of matrix metalloproteinases (32, 33), altered hematopoiesis (34), and blunted expression of both intercellular adhesion molecule-1 and  $\beta_2$ -integrin (35). Furthermore, because gain of function was not demonstrated in this study (29), it is possible that macrophages must act in combination with other cell types to facilitate neutrophilic recruitment to the lung. This process is seen in an *Escherichia coli* model of urosepsis, where expression of tlr4 on a combination of both stromal and hematopoietic cells is required for the clearance of whole bacteria (36). Therefore, it remains unclear which cell type or combination of cell types are required to detect LPS in the airway and initiate the signals required to recruit neutrophils to the airspace.

To address directly whether tlr4 expression in either hematopoietic cells or nonhematopoietic cells is sufficient for the biological response to inhaled LPS, we generated bone marrow chimeric mice using wild-type and tlr4-deficient mice. Our findings demonstrate a critical role of tlr4 expression on hematopoietic cells and, specifically, alveolar macrophages for the biological response to inhaled LPS.

(Received in original form July 22, 2004; accepted in final form December 13, 2004)

Supported by grants from the National Institute of Environmental Health Sciences (ES12717, ES7031, ES12496, ES11375, ES07498, and ES011961), the National Heart, Lung, and Blood Institute (HL74538), the Department of Veterans Affairs, and GlaxoSmithKline.

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This article has an online supplement, which is accessible from this issue's table of contents at [www.atsjournals.org](http://www.atsjournals.org)

Am J Respir Crit Care Med Vol 171, pp 806–813, 2005

Originally Published in Press as DOI: 10.1164/rccm.200407-953OC on December 23, 2004  
Internet address: [www.atsjournals.org](http://www.atsjournals.org)

## METHODS

### Inbred Mice

Genetically engineered *tlr4*-deficient mice, provided by Dr. Takeuchi from Osaka University (18), were backcrossed onto a C57BL/6 background for at least eight generations. Experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at Duke University Medical Center and performed in accordance with the standards established by the U.S. animal welfare acts.

### Hematopoietic Cell Transplantation and Adoptive Transfer

To examine the relative importance of *tlr4* expression in hematopoietic and nonhematopoietic cells in the response to inhaled LPS, we created bone marrow chimeric mice. Mice expressing *tlr4* in hematopoietic cells but not structural cells (H+/S−) were generated by transferring bone marrow from wild-type mice (CD45.1) into irradiated *tlr4*-deficient mice (CD45.2). Conversely, mice expressing *tlr4* in structural cells but not hematopoietic cells (H−/S+) were generated by transferring bone marrow from *tlr4*-deficient mice (CD45.2) into irradiated wild-type mice (CD45.1). Wild-type (H+/S+) and knock-out (H−/S−) control groups were generated by transferring bone marrow from wild-type mice (CD45.1) to wild-type mice (CD45.2), and from *tlr4*-deficient mice (CD45.2) to *tlr4*-deficient mice (CD45.2). These chimeric animals were created by lethal irradiation of 6- to 8-week-old recipient female mice with 10.5 Gy. Within 4 hours after irradiation, the mice were injected intravenously with  $4 \times 10^6$  donor bone marrow cells. Engraftment was confirmed by flow cytometry in the peripheral blood at 5 to 6 weeks after transplantation and in bronchial lavage cells at the time of killing. All animals were phenotyped 8 to 10 weeks after transplantation. Eight to nine animals were included in each experimental group. For animals undergoing serial depletion, a repeat bone marrow transplant was performed 3 weeks after the initial transplantation. For the serial transplantation experiments, nine animals per group were included in the serial transplants and four animals per group were singly transplanted as controls. All groups were phenotyped 8 to 10 weeks after the initial transplantation. Adoptive transfer of alveolar macrophages was achieved by harvesting whole lung lavage from five unexposed donor animals per recipient and pooling cells. A total of  $2 \times 10^5$  cells, which comprised more than 94% alveolar macrophages, was then intratracheally instilled in recipient animals anesthetized with isoflurane. Five adoptive transfer recipient animals per group were then phenotyped 48 hours after instillation to their response to inhaled endotoxin.

### Flow Cytometry

**Absolute count of lymphocyte and lymphocyte subsets in peripheral blood.** Fifty microliters of heparinized peripheral blood were stained with monoclonal antibodies for 15 minutes at room temperature. The stained whole blood samples were then processed in a Multi-Q-Prep (Coulter, Miami, FL) to lyse red blood cells. Fifty microliters of Flow-Count fluorospheres (Coulter) were added before flow cytometric analysis. The stained cells were analyzed using Coulter EPICS XL equipped with System II software.

**Bronchial lavage cells and parenchyma.** A total of  $1 \times 10^6$  fresh cells was obtained from whole lung lavage and stained with monoclonal antibodies. Whole lung samples were digested with collagenase and stained with monoclonal antibodies. Cells were analyzed using FACS Vantage SE (BD BioSciences, San Jose, CA), and counts were calculated automatically by FlowJo software (Tree Star, Inc., Ashland, OR).

### LPS Aerosol Challenge

LPS was purchased as lyophilized, purified *E. coli* 0111:B4 (Sigma, St. Louis, MO). LPS aerosol was generated and monitored as previously described. We have determined the minimal dosage required to initiate an inflammatory response in the lower respiratory tract similar to that experienced by grain mill workers during a typical 8-hour workday. The mean concentrations of LPS aerosol generated in these experiments was  $7.49 \mu\text{g}/\text{m}^3$  (3.87–11.0  $\mu\text{g}/\text{m}^3$ ) as measured by limulus amoebocyte lysate (BioWhittaker, Walkerville, MD). Airway pressure time index was performed 4 to 8 hours after LPS challenge. Bronchial hyperactivity and neutrophilic inflammation have been previously demonstrated

at this time point (37–40). Mice were killed immediately after the completion of assessment of pulmonary function.

### Whole Lung Lavage and Myeloperoxidase Assay

Whole lung lavage was performed as previously described (19, 37, 38, 41). Cell-free lavage supernatants were stored at  $-70^\circ\text{C}$ . ELISA, to evaluate protein concentrations of tumor necrosis factor  $\alpha$ , interleukin  $1\beta$ , and monocyte chemotactic protein-1 were purchased from R&D Systems (Minneapolis, MN). Luminex (BioRad, Hercules, CA) was used to evaluate protein concentrations of keratinocyte-derived chemokine, interleukin 6, and macrophage inflammatory protein-1 $\alpha$  with a commercially available immunoassay (Linco Research, Inc., St. Charles, MO). Concentrations of proteins in lavage fluid were reported as pg/ml. Myeloperoxidase concentrations were evaluated on homogenized whole lung with a commercially available assay and reported as U/g tissue (CytoStore, Calgary, AB, Canada).

### Airway Physiology

**Whole body plethysmography.** Unrestrained whole body plethysmography (Buxco Electronics, Inc., Troy, NY) was conducted (42–44), and measurements were obtained at baseline and after stimulation with inhaled methacholine (0, 5, 10, and 20 mg/ml), as previously described (37, 41).

**Airway pressure time index.** Mice were anesthetized with pentobarbital sodium (60 mg/kg) intraperitoneally. Airway pressure was measured by a differential pressure transducer connected to the side port of a surgically inserted tracheostomy cannula used to ventilate the animals with 6 to 8 ml/kg  $\text{V}_\text{T}$  at 125 breaths/minute. Neuromuscular blockade was accomplished with doxacurium chloride (0.25 mg/kg) and assessed by spontaneous respiratory efforts. Intravenous methacholine (25, 100, and 250  $\mu\text{g}/\text{kg}$ ) was administered into the jugular vein.

### Statistics

Data are expressed as mean  $\pm$  SEM, and the underlying characteristics of the distribution were evaluated using standard techniques (45). Significant differences between groups were identified by analysis of variance. Individual comparisons between groups were confirmed by the Student's *t* test or, when appropriate, the Mann-Whitney U test (45). Statistical calculations were performed using SPSS software (SPSS, Inc., Chicago, IL). A two-tailed *p* value of less than 0.05 was considered statistically significant.

## RESULTS

### Generation of Bone Marrow Chimeric Mice

The complete ablation of hematopoietic cells by irradiation and successful reconstitution was essential to the success of these experiments. To determine the extent of engraftment of various cell types, we used two different substrains of C57BL/6 mice having a polymorphism in the cell surface protein CD45 (CD45.1 and CD45.2). Flow cytometry analysis of peripheral blood revealed that after a single irradiation and bone marrow transplantation, peripheral engraftment was high in all peripheral blood cell types, including the following: CD4 T lymphocytes (90.4%, range 83.2–94.5%), CD8 T lymphocytes (86.7%, range 74.2–94.2%), B lymphocytes (99.9%, range 99.8–100%), and all leukocytes (95.7%, range 94.4–99.2%; Figure 1). Alveolar engraftment of F4/80-negative cells (primarily neutrophils) was 99.1% (range 85.5–99.9%; Figures 2A and 2B). A second transplant was performed to evaluate the characteristics of macrophage populations in unexposed animals (data not shown). In this group, a high engraftment was achieved in both the lavage cells (88%) and lung parenchyma (67%). Furthermore, we did not observe any new populations of antigen-presenting cells enter the lung as a result of bone marrow transplantation when evaluating surface expression by flow cytometry of MHCII, CD11c, or CD11b as compared with nonirradiated wild-type animals.

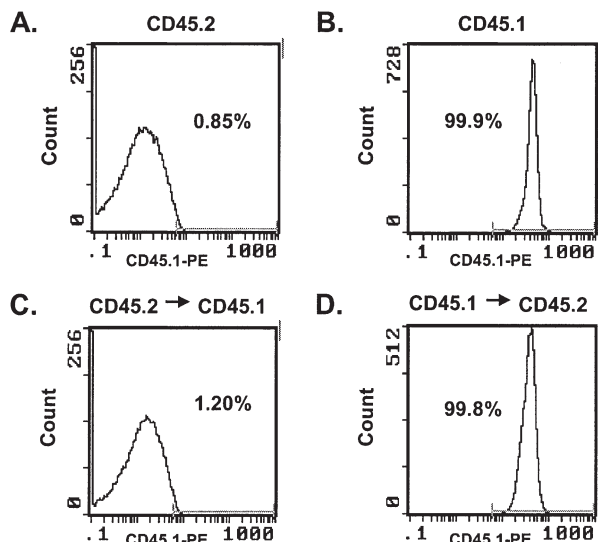


**Figure 1.** Peripheral engraftment. Whole blood was evaluated for leukocyte engraftment by CD45.1 expression. Antibodies used in these experiments are robust for negative (A) and positive (B) controls. Lymphocyte engraftment was confirmed in H<sup>+</sup>/S<sup>+</sup> (C) and H<sup>+</sup>/S<sup>-</sup> (D) animals.

#### Hematopoietic Expression of tlr4 Is Sufficient for LPS-induced Neutrophil Recruitment to the Lower Respiratory Tract

As expected, tlr4 was required for a robust inflammatory response, with tlr4-deficient mice having 17-fold fewer total cells

( $p < 0.005$ ) and 400-fold fewer neutrophils ( $p < 0.005$ ) than wild-type mice (Figure 3). Irradiation of the mice did not significantly affect neutrophil recruitment because irradiated wild-type mice reconstituted with wild-type bone marrow (H<sup>+</sup>/S<sup>+</sup>) had responses that were indistinguishable from nonirradiated wild-type mice. Similarly, irradiated tlr4-deficient mice reconstituted with tlr4-deficient bone marrow (H<sup>-</sup>/S<sup>-</sup>) had more than 7-fold fewer total cells ( $p < 0.001$ ) and 250-fold fewer neutrophils ( $p < 0.001$ ) than H<sup>+</sup>/S<sup>+</sup> mice. Furthermore, the responses of tlr4-deficient mice reconstituted with wild-type bone marrow (H<sup>+</sup>/S<sup>-</sup>) were indistinguishable from those of wild-type mice. This finding indicates that hematopoietic expression of tlr4 (H<sup>+</sup>/S<sup>-</sup>) is sufficient to completely reconstitute the inflammatory response to inhaled endotoxin. Conversely, wild-type mice receiving tlr4-deficient bone marrow (H<sup>-</sup>/S<sup>+</sup>) had a greater than 2.5-fold reduction in total cells ( $p < 0.01$ ) and a 4-fold reduction in neutrophils ( $p < 0.003$ ) when compared with the H<sup>+</sup>/S<sup>+</sup> chimeras, further demonstrating the importance of tlr4-expressing hematopoietic cells in the response to inhaled LPS. These H<sup>-</sup>/S<sup>+</sup> chimeras were not devoid of a biological response, however, and had more total cells ( $p = 0.01$ ) and 63-fold more neutrophils ( $p = 0.002$ ) than H<sup>-</sup>/S<sup>-</sup> chimeric mice, which are devoid of any tlr4 expression.

To evaluate whether the decreased inflammatory response to LPS in H<sup>-</sup>/S<sup>+</sup> mice merely represented lung sequestration of neutrophils, whole lung homogenates were evaluated in lavage lungs for myeloperoxidase activity. H<sup>+</sup>/S<sup>+</sup> and H<sup>+</sup>/S<sup>-</sup> chimera demonstrated high concentrations of myeloperoxidase activity,  $27.2 (\pm 3.7)$  and  $25.2 (\pm 2.1)$ , respectively. H<sup>-</sup>/S<sup>+</sup> and H<sup>-</sup>/S<sup>-</sup> had significantly lower myeloperoxidase activity,  $17.4 (\pm 2.4)$  and  $13.9 (\pm 2.0)$ , respectively. Similar to the whole lung lavage, animals expressing bone marrow-derived tlr4 demonstrated

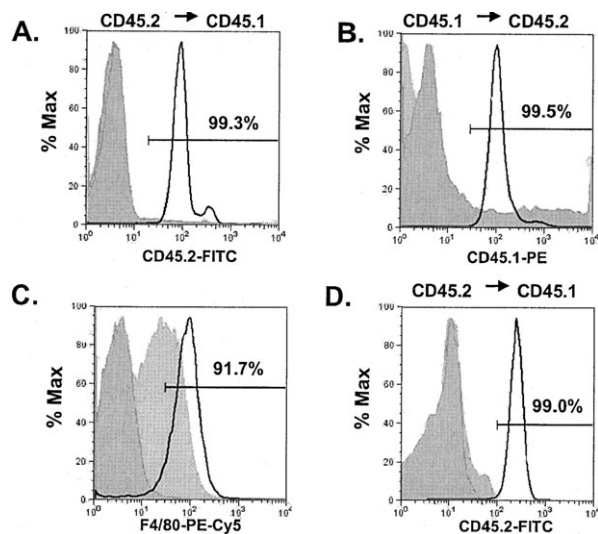


**Figure 2.** Pulmonary engraftment. Whole lung lavage was evaluated for engraftment by both CD45.1 and CD45.2 expression. Neutrophils account for more than 91% of cells in bronchoalveolar lavage fluid (BALF) after exposure to LPS. Nonmacrophages (F4/80-negative) cells were evaluated for engraftment after LPS exposure. When compared with either isotype (light gray) or autofluorescence (dark gray), we can determine the level of CD45.2 (A) and CD45.1 (B) engraftment. In unexposed, double-transplanted animals, macrophages account for more than 90% of cells in BALF. Macrophages (F4/80-positive) were identified, despite some overlap with isotype controls (light gray; C). Percentage of alveolar macrophage engraftment was determined by presence of CD45.2 as compared with CD45.1 within F4/80-positive cells (D).

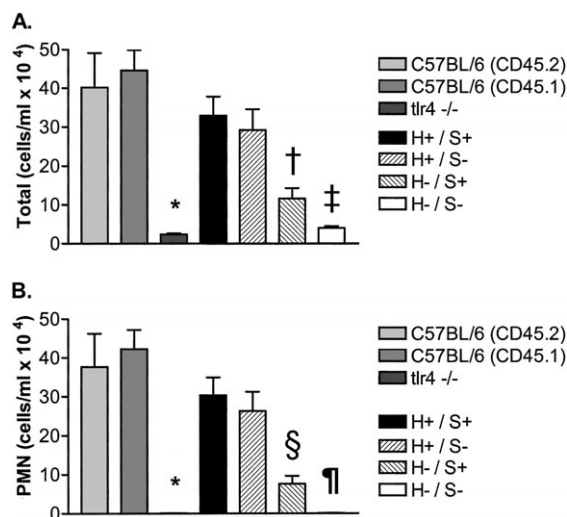


**Figure 3.** Hematopoietic-derived tlr4 is sufficient for neutrophil recruitment into the lung in response to inhaled endotoxin after single bone marrow transplantation. Total cells (A) and neutrophils (B) in BALF were evaluated. Tlr4-deficient remained unresponsive to inhaled endotoxin, when compared with wild-type control animals, and demonstrated significantly fewer total cells (\* $p < 0.005$ ) and neutrophils (\* $p < 0.005$ ). The ability to recruit total cells and neutrophils into the lung was completely reconstituted in H<sup>+</sup>/S<sup>-</sup> chimera mice. H<sup>-</sup>/S<sup>+</sup> animals demonstrate significantly fewer total cells († $p < 0.01$ ) and neutrophils (§ $p < 0.003$ ) when compared with H<sup>+</sup>/S<sup>+</sup>. H<sup>-</sup>/S<sup>+</sup> animals demonstrate more total cells (‡ $p = 0.01$ ) and neutrophils (§ $p = 0.002$ ) than H<sup>-</sup>/S<sup>-</sup> animals. PMN = neutrophils.

significantly more myeloperoxidase activity when compared with chimeras devoid of hematopoietic *tlr4* ( $p < 0.01$ ), again demonstrating the importance of *tlr4*-expressing hematopoietic cells in the response to inhaled LPS.

#### Tlr4-expressing Lung Structural Cells Play a Minor Role in LPS-induced Neutrophil Recruitment to the Lower Respiratory Tract

At least two possibilities could explain the increased concentration of neutrophils in H-/*S*+ animals compared with H-/*S*- mice. Although *tlr4* expression in nonhematopoietic cells might participate in the biological response to inhaled LPS, it is also possible that incomplete ablation of hematopoietic *tlr4*<sup>+/+</sup> expression in H-/*S*+ animals (e.g., residual lung parenchymal macrophages bearing *tlr4*) accounted for at least part of their response to inhaled LPS. If so, mice undergoing a second serial irradiation and bone marrow transplant would be expected to have a reduced response compared with single-irradiated H-/*S*+ mice. We therefore performed a second irradiation and bone marrow transplant of H-/*S*+ mice and analyzed these mice by flow cytometry to determine their extent of reconstitution. Virtually 100% of peripheral leukocytes in these double-transplanted mice were of donor origin including the following: CD4 T cells (99.8%, range 99.3–99.9%), CD8 T cells (99.9%, range 99.6–100%), and B cells (99.7%, range 99.4–100%), and all leukocytes (99.6%, range 99.4–100%). Importantly, flow cytometry analysis of whole lung lavage in unexposed double-transplanted animals revealed that donor marrow-derived cells constituted approximately 99.9% (range 99.8–100%) of the alveolar macrophage population (F4/80-positive; Figures 2C and 2D).

The higher level of engraftment seen in double-transplanted mice compared with single-transplanted mice provided a superior model to test the requirement of *tlr4* expression in non-bone marrow-derived cells. Serial bone marrow transplantation on its own did not affect the ability of H+/*S*+ animals to recruit neutrophils into the airway. However, after double transplantation of H-/*S*+, there was a sixfold reduction in both total cells ( $p < 0.001$ ; Figure 4A) and neutrophils ( $p < 0.001$ ; Figure 4B) compared with double-irradiated H+/*S*+ mice. Although markedly attenuated, the ability to recruit inflammatory cells with structural expression of *tlr4* was not completely ablated even in these double-irradiated H-/*S*+ mice. When compared with double-irradiated H-/*S*- mice, the double-irradiated H-/*S*+ mice had a small (9%) but significant ( $p < 0.01$ ) increase in total cells and a 2.5-fold increase in neutrophils ( $p < 0.05$ ). These findings suggest that expression of *tlr4* in nonhematopoietic cells may play a minor role in recruiting neutrophils into the lung after inhaled LPS.

#### Production of Proinflammatory Cytokines and Chemokines Are Dependent on Hematopoietic *tlr4*<sup>+/+</sup>

After stimulation with LPS, many cell types in the lung can produce cytokines and chemokines. These molecules are believed to contribute to neutrophil recruitment and may also contribute to airway hyperreactivity. To determine whether *tlr4* expression in hematopoietic or nonhematopoietic cells is required for cytokine production, we measured levels of several different cytokines in the whole lung lavage fluid of LPS-challenged single-transplanted mice. Wild-type animals and H+/*S*+ chimeras had much higher levels of proinflammatory cytokines than *tlr4*-deficient animals and H-/*S*- mice, respectively (Table 1). Although C57BL/6 (CD45.1) animals demonstrated lower levels of many proinflammatory cytokines when compared with C57BL/6 (CD45.2) animals, this difference would minimize the reconstitution of cytokine production in the H+/*S*- chimera mice given that wild-type donor

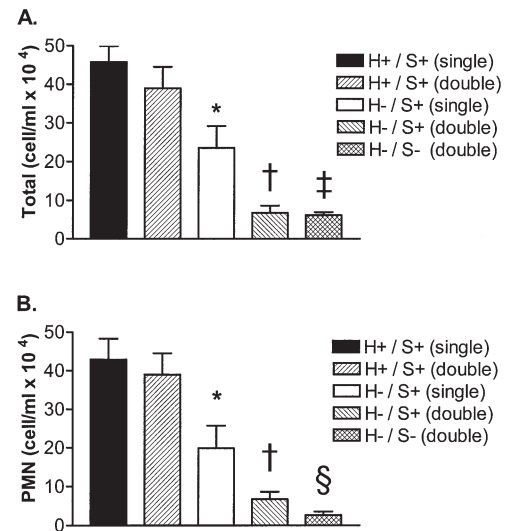


**Figure 4.** Increased engraftment led to attenuation of inflammatory response in H-/*S*+. Serial irradiation and reconstitution led to increased level of engraftment. Total cells (A) and neutrophils (B) in BALF were evaluated. There were no differences in total cells or neutrophils in H+/*S*+ dependent on serial transplantation. Single-transplanted H-/*S*+ had a reduction in total cells (\* $p < 0.05$ ) and neutrophils (\* $p < 0.05$ ), when compared with H+/*S*+. Double transplantation of H-/*S*+ results in fewer total cells († $p < 0.001$ ) and neutrophils († $p < 0.001$ ) when compared with H+/*S*+. Double transplantation of H-/*S*+ resulted in fewer total cells ( $p = 0.02$ ) and neutrophils ( $p = 0.02$ ) when compared with single-irradiated H-/*S*+. Double-transplanted H-/*S*+ animals had persistent increases in total cells (§ $p < 0.01$ ) and neutrophils (§ $p < 0.04$ ) when compared with double-transplanted H-/*S*- animals.

animals were C57BL/6 (CD45.1). Concentrations of cytokines in H+/*S*- animals were greater than or equal to either wild-type control or H+/*S*+ mice, demonstrating that hematopoietic expression of *tlr4* is sufficient to reconstitute these potent mediators of inflammation. By contrast, H-/*S*+ animals had significant reductions in all measured proinflammatory markers when compared with H+/*S*+ controls ( $p < 0.01$ ). Furthermore, the concentration of proinflammatory cytokines in the H-/*S*+ group were only barely detectable, even though there were significant numbers of neutrophils in lungs of these mice. Therefore, cytokine production in response to inhaled LPS is even more dependent on hematopoietic expression of *tlr4* than is neutrophil recruitment.

#### Hematopoietic *tlr4*<sup>+/+</sup> Is Sufficient for the Development of Airway Hyperresponsiveness in Response to Inhaled LPS

Although we found that hematopoietic-derived *tlr4* was sufficient for both neutrophil recruitment into the lung and production of proinflammatory cytokines, it remained to be determined whether expression of *tlr4* in these cells was sufficient for the development of airway hyperreactivity. We therefore measured changes in tracheal pressure of experimental mice after LPS inhalation and methacholine challenge (Figure 5). Wild-type animals had significant increases in airway hyperreactivity following inhalation of LPS when compared with *tlr4*-deficient mice ( $p < 0.005$ ). There were no significant differences between irradiated and non-irradiated control animals. H+/*S*+ chimera demonstrated increased hyperresponsiveness when compared with H-/*S*- ( $p = 0.05$ ). H+/*S*- chimeras had levels of hyperresponsiveness much greater than either *tlr4*-deficient mice or H-/*S*- chimeric mice ( $p = 0.003$ ; Figure 5) and were not significantly different than

**TABLE 1. HEMATOPOIETIC-DERIVED *tlr4* IS SUFFICIENT FOR EXPRESSION OF PROINFLAMMATORY CYTOKINES AFTER SINGLE BONE MARROW TRANSPLANTATION**

|                            | TNF- $\alpha$          | IL-6                    | MIP-1 $\alpha$          | KC                      | IL-1 $\beta$             | MCP-1                   |
|----------------------------|------------------------|-------------------------|-------------------------|-------------------------|--------------------------|-------------------------|
| C57BL/6 (CD45.2)           | 470 $\pm$ 53           | 196 $\pm$ 36            | 37 $\pm$ 13             | 178 $\pm$ 35            | 182 $\pm$ 73             | 65 $\pm$ 17             |
| C57BL/6 (CD45.1)           | 243 $\pm$ 62           | 94 $\pm$ 13             | 10 $\pm$ 7              | 48 $\pm$ 27             | 111 $\pm$ 27             | 78 $\pm$ 13             |
| <i>tlr4</i> <sup>-/-</sup> | 0* <sup>†</sup>        | 0 $\pm$ 0* <sup>†</sup> | 1 $\pm$ 0* <sup>†</sup> | 1 $\pm$ 0* <sup>†</sup> | 12 $\pm$ 8* <sup>†</sup> | 0 $\pm$ 0* <sup>†</sup> |
| H+/S+                      | 285 $\pm$ 84           | 281 $\pm$ 64            | 20 $\pm$ 14             | 52 $\pm$ 16             | 149 $\pm$ 37             | 35 $\pm$ 8              |
| H+/S-                      | 409 $\pm$ 115          | 241 $\pm$ 50            | 41 $\pm$ 16             | 82 $\pm$ 24             | 356 $\pm$ 82             | 241 $\pm$ 57            |
| H-/S+                      | 0 $\pm$ 0 <sup>‡</sup> | 13 $\pm$ 6 <sup>‡</sup> | 2 $\pm$ 1 <sup>‡</sup>  | 5 $\pm$ 2 <sup>‡</sup>  | 33 $\pm$ 10 <sup>‡</sup> | 9 $\pm$ 4 <sup>‡</sup>  |
| H-/S-                      | 0 $\pm$ 0 <sup>‡</sup> | 0 $\pm$ 0 <sup>‡</sup>  | 0 $\pm$ 0 <sup>‡</sup>  | 1 $\pm$ 0 <sup>‡</sup>  | 3 $\pm$ 2 <sup>‡</sup>   | 0 $\pm$ 0 <sup>‡</sup>  |

Definition of abbreviations: IL = interleukin; KC = keratinocyte-derived chemokine; MCP-1 = monocyte chemoattractant protein-1; MIP-1 $\alpha$  = macrophage inflammatory protein-1 $\alpha$ ; TNF- $\alpha$  = tumor necrosis factor  $\alpha$ .

Data expressed as mean pg/ml ( $\pm$  range). Comparisons were made with nonparametric Mann-Whitney U test.

\*  $p < 0.001$  compared with C57BL/6.CD45.2.

<sup>†</sup>  $p < 0.01$  compared with C57BL/6.CD45.1.

<sup>‡</sup>  $p < 0.01$  compared with H+/S+.

H+/S+ or wild-type control mice. The airway response in H-/S+ animals was only minimally attenuated and was not statistically different than H+/S+ control animals. Collectively, these data support the hypothesis that expression of *tlr4* in hematopoietic cells or lung structural cells is sufficient to reconstitute the airway response to inhaled LPS.

#### Alveolar Macrophage Expression of *tlr4* Is Sufficient for Neutrophil Recruitment after Exposure to Inhaled LPS but Not Airway Hyperreactivity

The experiments with bone marrow chimeric mice demonstrated that hematopoietic expression of *tlr4* was sufficient for neutrophil recruitment, cytokine production, and airway hyperreactivity. However, these experiments were unable to identify the specific hematopoietic cell type whose expression of *tlr4* was necessary for the biological response to inhaled endotoxin. Alveolar macrophages, which are the predominant cell type in the airway of unchallenged mice, were a plausible candidate. To determine whether *tlr4* expression in alveolar macrophages was sufficient for the biological response to LPS, we adoptively transferred wild-type alveolar macrophages intratracheally into *tlr4*-deficient animals, and then exposed these recipient mice to aerosolized LPS. The transfer of wild-type alveolar macrophages into *tlr4*-deficient mice resulted in a 3.9-fold increase in total cells ( $p < 0.01$ ; Figure 6A) and a more than 13-fold increase in the number of neutrophils ( $p < 0.01$ ; Figure 6B) when compared with similarly challenged *tlr4*-deficient mice receiving *tlr4*-deficient macrophages. Although we did not recruit the same con-

centration of neutrophils as in wild-type controls, this observation demonstrates that expression of *tlr4* on alveolar macrophages is sufficient to restore the neutrophilic inflammatory response to inhaled LPS in *tlr4*-deficient mice. In addition, these mice failed to develop LPS-induced airway hyperreactivity and were indistinguishable in this regard from *tlr4*-deficient animals (Fig-

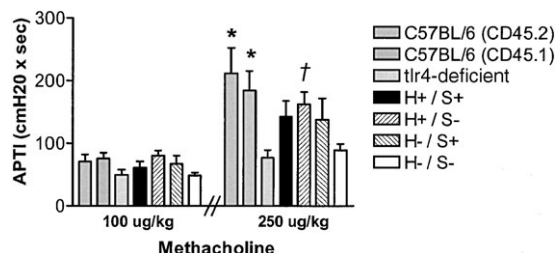


**Figure 5.** Hematopoietic-derived *tlr4* was sufficient for increased airway hyperreactivity in response to inhaled endotoxin. After methacholine challenge, wild-type animals had increased airway pressure time index (APTI) when compared with *tlr4*-deficient animals (\* $p < 0.005$ ). There were no observable differences between H-/S- and *tlr4*-deficient animals. H+/S- had increased response to methacholine when compared with H-/S- (\* $p = 0.003$ ).

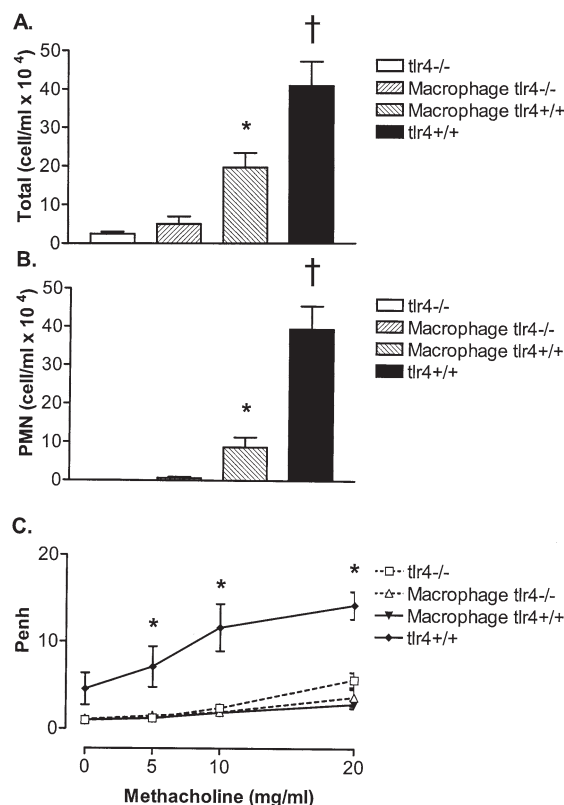


**Figure 6.** Alveolar macrophage-derived *tlr4* was sufficient for neutrophil recruitment into the lungs. (A, B) Adoptive transfer of unchallenged macrophages expressing *tlr4* reconstituted the ability to recruit total cells (\* $p < 0.01$ ) and neutrophils (\* $p < 0.01$ ) when compared with transfer of *tlr4*-deficient macrophages. Wild-type animals had increased total cells (\* $p < 0.001$ ) and neutrophils (\* $p < 0.001$ ) when compared with transfer of *tlr4*-deficient macrophages. (C) Wild-type animals had increased airway obstruction (\* $p < 0.05$ ) when compared with *tlr4*-deficient. Animals with macrophage expression of *tlr4* were no different than *tlr4*-deficient. Penh = enhanced pause.

ure 6C). Furthermore, mice with alveolar macrophage expression of *tlr4* alone did not produce significant concentrations of tumor necrosis factor  $\alpha$ , macrophage inflammatory protein-1 $\alpha$ , or interleukin 6, despite their ability to recruit neutrophils into the airspace (Table 2). These findings demonstrate that, unlike neutrophil recruitment, the development of airway hyperreactivity and the production of proinflammatory cytokines/chemokines may require interaction with bone marrow-derived cells, other than macrophages, that express *tlr4*.

## DISCUSSION

Our findings indicate that expression of *tlr4* in hematopoietic cells alone is sufficient to fully reconstitute the biological response to inhaled LPS, as measured by neutrophil recruitment, cytokine/chemokine production, and airway hyperreactivity. These findings demonstrate that expression of *tlr4* in non-bone marrow-derived cells, such as epithelial cells, endothelial cells, smooth muscle cells, or fibroblasts, is not required for the inflammatory response to inhaled LPS. In contrast, the expression of *tlr4* in lung structural cells appears to be sufficient for the development of LPS-induced airway hyperreactivity. Moreover, our findings demonstrate the importance of the alveolar macrophage in promoting the inflammatory (but not the physiologic) response to inhaled LPS.

The site of the stimulus appears to be critical in determining the cell types that require *tlr4* expression for either sequestration of neutrophils in the lung or movement of neutrophils from the vascular space to the airspace. Our finding that *tlr4* expression in structural cells is not required for movement of neutrophils from the vascular space to the airspace after inhalation of LPS contrasts with the previously demonstrated requirement of *tlr4* expression by endothelial cells for neutrophil sequestration in the lung after a systemic challenge with LPS (27). Our data suggest that, after LPS inhalation, *tlr4*-expressing alveolar macrophages produce and release critical biological mediators, which could act either locally or systemically, resulting in the recruitment of neutrophils into the lower respiratory tract. Factors produced by macrophages could indirectly activate other cell types, including epithelia and endothelia, independent of *tlr4* expression on those cell types. Our observations are not inconsistent with previous observations by Skerrett and coworkers (28), highlighting the critical role of airway epithelia in the pulmonary inflammatory response. Although our results demonstrate that neutrophil recruitment in response to inhaled LPS is independent of *tlr4* expression on structural cells, we propose a cooperative model where macrophage-derived factors can act indirectly on either airway epithelia or endothelium independent of *tlr4* expression. There is ample evidence that macrophages both express *tlr4* and have a robust response to LPS (18). Local or

systemic factors altered by macrophage activation could play a role in neutrophil recruitment after exposure to inhaled LPS, including the following: cytokines, chemokines, matrix proteins, or metalloproteinases (46). It is known that cytokines (47), chemokines (48), and metalloproteinases (49) can effect neutrophil migration. Although the specific factors controlling the migration of neutrophils into the lung in this model remain undetermined, our data suggest that macrophages alone can provide the initial critical signals required to stimulate movement of neutrophils from the vascular space to the airspace. It remains plausible that endothelial cells are capable of initiating similar signaling pathways required for neutrophil sequestration in response to systemic LPS. Although we observed striking differences between the types of cells required dependent on the site of LPS exposure, there is at least one similarity: with either inhaled or systemic LPS exposure, neutrophil migration was independent of neutrophil expression of *tlr4* (27). This finding is highlighted in H-/S+ animals where recruited neutrophils are *tlr4*-deficient and after adoptive transfer of *tlr4*+/+ macrophages where recruited neutrophils are *tlr4*-deficient. Although, *tlr4*-deficient neutrophils can be recruited into the airway, we speculate that neutrophil expression of *tlr4* contributes to the production of proinflammatory cytokines and airway hyperreactivity in response to inhaled LPS.

Previous studies from our laboratory have demonstrated that inhaled LPS results in the release of cytokines (tumor necrosis factor  $\alpha$  and interleukin 1 $\alpha$ ) and chemokines (macrophage inflammatory protein-2) produced by inflammatory cells in the lung (50). This study found that production of these cytokines and chemokines is dependent on the expression of *tlr4* on hematopoietic cells and not lung structural cells. We speculate that neutrophil-derived *tlr4* plays an important role in the production of proinflammatory cytokines and chemokines for the following reasons: First, it is known that neutrophils both normally express *tlr4* and respond avidly to LPS (21). Second, despite the ability of single-transplanted H-/S+ animals to recruit *tlr4*-deficient neutrophils into the airway, these mice produce nearly undetectable levels of proinflammatory cytokines and chemokines. Third, adoptive transfer of macrophages expressing *tlr4* alone was also sufficient to recruit *tlr4*-deficient neutrophils, but the level of cytokines remained similar to *tlr4*-deficient animals. Although very low levels of cytokines in each of these groups could result from lower absolute levels of neutrophils in the airway, these observations support the importance of neutrophil expression of *tlr4* in the production of proinflammatory markers in mouse lungs in response to inhaled LPS.

The biological mechanisms required for neutrophil recruitment into the lung appear to be independent of those required for the development of airway hyperreactivity. Previously, our group has observed a *tlr4*-dependent phenotype in mice that is divergent between neutrophilic inflammation and airway hyperreactivity after exposure to ozone (19). The results presented in this study indicate that, although macrophage-derived *tlr4* alone can induce movement of the neutrophil from the vascular space to the airspace, this biological response is not sufficient to enhance airway hyperreactivity. From our bone marrow chimeras, it appears that *tlr4* expression by a combination of hematopoietic cells is sufficient to develop airway hyperresponsiveness in response to inhaled LPS. Taken together, we speculate that neutrophils expressing *tlr4* can contribute to both the production of cytokine/chemokines and the development of airway hyperreactivity. Although we also demonstrated that *tlr4* expression by structural lung cells is sufficient to induce airway hyperresponsiveness after inhalation of LPS, the mechanisms are not entirely obvious. Unlike neutrophil recruitment to the lung, it remains likely that *tlr4* expression by a combination of cell types is re-

**TABLE 2. ADOPTIVE TRANSFER OF ALVEOLAR MACROPHAGE-DERIVED *tlr4* AND PROINFLAMMATORY CYTOKINES**

|                            | TNF- $\alpha$ | IL-6            | MIP-1 $\alpha$ |
|----------------------------|---------------|-----------------|----------------|
| C57BL/6                    | 132 $\pm$ 3   | 143.8 $\pm$ 1.0 | 12 $\pm$ 5     |
| Macrophage <i>tlr4</i> +/+ | 2 $\pm$ 0*    | 8 $\pm$ 5*      | 0 $\pm$ 0*     |
| Macrophage <i>tlr4</i> -/- | 2 $\pm$ 0*    | 8 $\pm$ 8*      | 0 $\pm$ 0*     |
| <i>tlr4</i> -/-            | 2 $\pm$ 0*    | 0 $\pm$ 0*      | 0 $\pm$ 0*     |

Definition of abbreviations: IL = interleukin; MIP-1 $\alpha$  = macrophage inflammatory protein-1 $\alpha$ ; TNF- $\alpha$  = tumor necrosis factor- $\alpha$ .

Data expressed as mean pg/ml ( $\pm$  range). Comparisons were made with non-parametric Mann-Whitney U test.

\*p < 0.05 compared with C57BL/6.

quired for the development of airway hyperresponsiveness after exposure to inhaled LPS.

In summary, our results indicate that movement of neutrophils from the vascular space to the airspace and the release of cytokines/chemokines after inhalation of LPS are primarily dependent on the expression of *tlr4* by hematopoietic cells. Moreover, our results suggest that the alveolar macrophage alone is sufficient for neutrophil recruitment into the airspace. Although *tlr4* expression on endothelial cells is essential for sequestration of neutrophils in the lung after systemic exposure to LPS, *tlr4* expression on hematopoietic cells appears to be critical to the movement of neutrophils from the vascular space to the airspace after inhalation of LPS. These findings suggest that the pathophysiologic response to LPS is determined by specific site-dependent biological mechanisms.

**Conflict of Interest Statement:** J.W.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; B.J.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; D.M.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; K.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; M.D.G. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; D.N.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; D.A.S. has been reimbursed by Intermune between January 2000 and the present with \$16,000 per year.

**Acknowledgment:** The authors appreciate the support of Nelson Chao in the Division Cellular Therapy, Duke University Medical Center. John F. Whitesides performed flow cytometry on lung lavage cells at the Duke Human Vaccine Institute Flow Cytometry core facility, which is supported by the National Institutes of Health award AI-51445.

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