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Both Hemopoietic and Resident Cells Are Required for MyD88-Dependent Pulmonary Inflammatory Response to Inhaled Endotoxin

Nicolas Noulín,*† Valérie F. J. Quesniaux,* Silvia Schnyder-Candrian,* Bruno Schnyder,*§ Isabelle Maillet,*† Thomas Robert,* B. Boris Vargaftig,‡ Bernhard Ryffel,* and Isabelle Couillin²*†

Inhaled endotoxin induces an inflammatory response that contributes to the development and severity of asthma and other forms of airway disease. Here, we show that inhaled endotoxin-induced acute bronchoconstriction, TNF, IL-12p40, and KC production, protein leak, and neutrophil recruitment in the lung are abrogated in mice deficient for the adaptor molecule MyD88. Bronchoc- striction, inflammation, and protein leak are normal in Toll/IL-1R domain-containing adaptor inducing IFN-β-deficient mice. MyD88 is involved in TLR, but also in IL-1R-associated kinase 1-mediated IL-1R and -18R signaling. We exclude a role for IL-1 and IL-18 pathways in this response, as IL-1R1 and caspase-1 (ICE)-deficient mice develop lung inflammation while TLR4-deficient mice are unresponsive to inhaled LPS. Significantly, using bone marrow chimera, we demonstrate that both hemopoietic and resident cells are necessary for a full MyD88-dependent response to inhaled endotoxin; bronchoconstriction depends on resident cells while cytokine secretion is mediated by hemopoietic cells. The Journal of Immunology, 2005, 175: 6861–6869.

Inflammation of the airways, which is often associated with life-threatening infection by Gram-negative bacteria or presence of endotoxin in the bioaerosol, is still a major cause of severe airway disease (1). Moreover, inhaled endotoxin may play an important role in the development and progression of airborne inflammation in asthma (2–5). Pathologic changes induced by endotoxin inhalation include bronchospasm, airflow obstruction, recruitment of inflammatory cells, injury of the alveolar epithelium, and disruption of pulmonary capillary integrity leading to protein-rich fluid leak in the alveolar space (6, 7). The majority of these pathologic features of human airway inflammation have also been observed in experimental lung injury models. In mice, aerogenic exposure to endotoxin or LPS, a major component of the outmost membrane of Gram-negative bacteria, induces pulmonary inflammation with recruitment and activation of macrophages and neutrophils in the airways, local TNF production, alveolar-capillary leak, and also a direct bronchoconstriction (8). TLR, and especially TLR4, plays a critical role in the response to LPS (9). Lung injury has been shown to be CD14 and TLR4-dependent in a murine sepsis model after systemic LPS administration (10, 11). Lungs are a complex compartmentalized organ with separate barriers, namely the alveolar-capillary barrier, the microvascular endothelium, and the alveolar epithelium. Systemic LPS administration induces neutrophil sequestration into the pulmonary microvasculature without passage into the lung tissues and bronchoalveolar space (12), whereas neutrophil recruitment upon aerogenic LPS exposure occurs in all airway compartments (8). The mechanisms of these responses are still poorly understood, and we analyzed the role of TLR signaling and the contribution of different cell types in response to aerogenic LPS.

We focused on the role of the common TLR and IL-1R adaptor molecule, the MyD88. Absence of MyD88 confers resistance to systemic endotoxin-induced shock (13), although there is evidence that LPS can use MyD88-independent signaling pathways (14). In particular, other adaptor proteins such as TIR domain-containing adaptor inducing IFN-β (TRIF)³ (15, 16) and TRIF-related adaptor molecule (TRAM) (17, 18) have been implicated in some responses to LPS resulting in IFN type I-dependent expression of costimulatory molecules. TRAM is thought to act as a link between TRIF and TLR4, like Toll/IR-1R domain-containing adaptor protein bridging MyD88 to TLR4. MyD88 and Toll/IL-1R domain-containing adaptor protein are involved in early activation of NF-κB and MAPK (19–22), whereas TRIF and TRAM are critical for late activation of NF-κB as well as the activation of IRF-3 (18, 23). A recent work on macrophages/DC suggests that no pathways other than MyD88-dependent or TRIF-dependent pathways exists in response to LPS in TLR4-mediated signaling (24), whereas a third pathway independent of TLR4 possibly exists (25). The responses in lung, a site of continuous exposure to environmental Ags, were believed to be different from these to less accessible sites (26, 27). Therefore, in view of the central role of the TLR adaptor molecule MyD88 and the site specificity of lung, we asked

³Abbreviations used in this paper: TRIF, Toll/IL-1R domain-containing adaptor inducing IFN-β; PenH, enhanced respiratory pause; AUC, area under the curve; MPO, myeloperoxidase; BALF, bronchoalveolar lavage fluid; WT, wild type; KO, knock-out; BMDM, bone marrow-derived macrophage; LTA, lipoteichoic acid; TRAM, TRIF-related adaptor molecule.

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to what extent inhaled endotoxin-induced lung injury is MyD88 dependent.

We demonstrate for the first time that MyD88 is indeed essential for the LPS-induced acute pulmonary inflammation response, whereas TRIF is dispensable. LPS-induced bronchoconstriction, pulmonary neutrophil sequestration, vascular leak, and TNF and IL-12p40 secretions were abrogated in MyD88−/− mice. Furthermore, using bone marrow chimera, we show that non-bone-marrow-derived resident cells, probably epithelial cells, are involved in sensing LPS to mediate the bronchoconstriction response, whereas the secretion of TNF and IL-12p40 in alveolar space is dependent on bone marrow-derived cells.

Materials and Methods

Mice

MyD88−/− (13), IL-1R1−/− (28), caspase-1−/− (29), TLR4−/− (30), TRIF−/− (16), and GFP transgenic mice were used (31). MyD88−/− and TLR4−/− were backcrossed 10 times on the C57BL/6 genetic background and 7 times for IL-1R1−/− and caspase-1−/− mice, respectively. All mice, including control C57BL/6 (MyD88+/+), were bred in our animal facility at the Transgenese Institute (Centre National de la Recherche Scientifique). For experiments, adult (6–10 wk old) animals were kept in sterile, isolated, ventilated cages. All animal experiments complied with the French Government’s ethical and animal experiment regulations.

Endotoxin administration and measurement of airway resistance

LPS (0.1–50 μg) from Escherichia coli (serotype O55: B5; Sigma-Aldrich) in saline or saline alone were given by the aerogenic route using nasal instillation in a volume of 50 μl under light ketamine-xylasine anesthesia. Alternatively, systemic effects of LPS were assessed after i.p. administration of 50 μg per mouse. The airway resistance was evaluated by whole-body plethysmography (8). Bronchoconstriction was investigated at several time points. Unrestrained conscious mice were placed in whole-body plethysmography chambers (EMKA Technologies). Mean airway bronchoconstriction readings, as assessed by enhanced respiratory pause (PenH), were observed over 3–6 h. PenH can be conceptualized as the phase shift of the thoracic flow and the nasal flow curves. Increased phase shift correlates with increased respiratory system resistance. PenH is calculated by the formula PenH = (Te/RT − 1) × PEFR/PEF, where Te is expiratory time, RT is relaxation time, PEF is peak expiratory flow, and PIF is peak inspiratory flow (32). Data are analyzed using Datanalyst software (EMKA Technologies) and expressed as mean ± SEM of PenH of individual mice per group. In all experiments performed, control administration of saline was without effect on PenH (data not shown).

Bronchoalveolar lavage fluid (BALF)

BALF was collected 3 or 24 h after LPS administration by cannulating the trachea under deep ketamine-xylasine anesthesia and washing the lung four times with 0.5 ml of saline at room temperature as described (32). The lavage fluid was centrifuged 10 min at 2000 rpm at 4°C and the supernatant frozen for cytokine content. The cell pellet was resuspended in PBS, counted in a hemocytometer chamber, and cytospin preparations were made using a Shandon cytocentrifuge (1000 rpm, 10 min). The cells were stained with Diff-Quick (Dade Behring). The supernatant was used for the measurement of cytokine and protein levels. TNF, IL-12p40, and KC were measured by enzyme-linked immunosorbent assay (R&D Duoset). For protein determination, Bradford stain was added to the supernatant as described by the manufacturer (Bio-Rad) using an OVA standard, and absorbance was measured at 595 nm (UVikon spectrophotometer; Kontron Zurich).

Microscopy and myeloperoxidase (MPO) activity in the lung

After bronchoalveolar lavage and lung perfusion, the mice were sacrificed. The lungs were fixed in 4% buffered formaldehyde for standard microscopic analysis; 3-μm sections were stained with H&E as described previously (32). Lung tissue MPO activity was evaluated as described (12). In brief, the right heart ventricle was perfused with saline to flush the vascular content, and lungs were frozen at −20°C until use. Lung was homogenized by polytron and centrifuged, and the supernatant was discarded. The pellets were resuspended in 1 ml of PBS containing 0.5% hexadecyltrimethyl ammonium bromide (HTAB) and 5 mM EDTA. After centrifugation, 50 μl of supernatants were placed in test tubes with 200 μl of PBS-HTAB-EDTA, 2 ml HBSS, 100 μl of o-dianisidine dihydrochloride (1.25 mg/ml), and 100 μl of H2O2, 0.05%. After 15 min of incubation at 37°C in an agitator, the reaction was stopped with 100 μl of Na2S, 1%. The MPO activity was determined as absorbance at 460 nm against medium.

Bone marrow transplantation to obtain mixed chimera

Recipient mice underwent a lethal total-body irradiation as reported before (33, 34). Fresh, unseparated bone marrow cells (2 × 106 per mouse) were injected into the lateral tail vein of the irradiated recipient mice 24 h after lethal irradiation. Blood was collected in EDTA containing tubes at regular intervals, and the hematological parameters were determined with a Technicon H6E analyzer. The reconstituted mice were used at 3 mo after bone marrow transplantation. Furthermore, in some experiments, we used GFP-expressing donor cells (31) to control the extent of reconstitution. The current protocol yielded >95% of blood circulating leukocytes of donor origin as assessed by green fluorescence of the peripheral blood using flow cytometry.

In vitro blood cell stimulation

Blood samples were obtained after cutting the end of the tail; 50 μl of blood was mixed immediately with 5 μl of heparin solution (250 IU/ml); 10 μl of this whole blood was then diluted in 190 μl of RPMI 1640 2.5% FCS medium with or without 0.1 μg/ml LPS. Cell supernatants were harvested after 18–24 h of stimulation and analyzed for TNF content.

Bone marrow-derived macrophage (BMDM) culture and stimulation in vitro

Primary BMDM were obtained from femoral bone marrow as described (33). In brief, cells from the femur were isolated and cultured at 106 cells/ml for 7 days in DMEM (Sigma-Aldrich) supplemented with 20% horse serum and 30% L929 cell-conditioned medium as a source of M-CSF. Three days after washing and reculturing in fresh medium, the cell preparation contained a homogenous population of >95% macrophages. The BMDM were plated in 96-well microculture plates (at 104 cells per well) and stimulated with LPS (E. coli, serotype O55: B5, at 100 ng/ml) and lipoteichic acid (LTA; Lunamed) at 10 μg/ml (35). Cell supernatants were harvested after 18–24 h of stimulation for TNF measurement and analyzed immediately or stored at −20°C.

Statistical analysis

Statistical evaluation of differences between the experimental groups was determined by Mann-Whitney U test for plethysmography experiments and Student’s t test for other data, using Prism software. Values of p < 0.05 were considered statistically significant.

Results

Absence of LPS-induced bronchoconstriction in MyD88−/− mice

Aerogenic administration of LPS induced within 90 min a direct bronchoconstriction in C57BL/6 (MyD88+/+) mice, whereas MyD88−/− mice were completely unresponsive to LPS. This bronchoconstriction increased for the next hours with a maximum PenH around 4 h, and then decreased toward basal level after 6 h (Fig. 1A). Bronchoconstriction was never observed in MyD88−/− mice even with high doses of LPS as represented by area under the curve (AUC) (Fig. 1B). Intranasal administration of NaCl did not induce bronchoconstriction in either mouse groups (data not shown). We further controlled for the systemic effects of LPS. Intraportal administration of 50 μg of LPS did not cause bronchoconstriction nor neutrophil recruitment in BALK because neutrophils were only sequestrated in the lung vasculature without any transpulmonary recruitment (data not shown). To test a contribution of the MyD88-independent signaling in LPS-induced bronchoconstriction, we tested TRIF-deficient mice (TRIF−/−) in the same conditions. TRIF−/− mice had normal bronchoconstriction response to aerogenic LPS (data not shown). Therefore, acute bronchoconstriction induced by intranasal instillation of LPS is strictly dependent on MyD88 signaling.
choconstriction response to aerogenic LPS (5072 h (Fig. 2)). Furthermore, no neutrophils were detectable in the lung (Fig. 2), similar to untreated controls (data not shown).

Absence of neutrophil recruitment into the lung in LPS-administered mice. Absence of LPS-induced bronchoconstriction in MyD88-/- mice. To test a contribution of the MyD88-independent signaling, we investigated the response to aerogenic LPS of IL-1R1-/- and caspase-1-/- mice, show a rapid bronchoconstriction response to aerogenic LPS (50 µg of LPS given by the intranasal route). The airway response is expressed as mean ± SEM of PenH (p < 0.001 from 90 to 360 min). B, AUC measured from 60 to 180 min after 1, 10, or 50 µg of intranasal LPS in MyD88-/- (■) and WT mice (□). Data are from one experiment representative of three independent experiments (n = 8 mice per group; *, p < 0.05).

Absence of neutrophil recruitment into the lung in LPS-challenged MyD88-/- mice

We then asked whether the absence of bronchoconstriction in MyD88-/- mice is associated with a reduced neutrophil recruitment into the lung. The micrographs of lung 24 h after LPS administration show abundant intra- and perivascular recruitment of neutrophils in the capillaries and alveolar space of wild-type (WT) mice, and its complete absence in MyD88-/- mice (Fig. 2A). To quantify neutrophil numbers, lung homogenates were assessed for MPO activity. Although WT mice showed an elevated MPO activity at 3 h, MyD88-/- mice had essentially no MPO activity in the lung (Fig. 2B), similar to untreated controls (data not shown).

The recruitment of neutrophils was not merely delayed in MyD88-/- mice because MPO activity did not increase at 24 and 72 h (Fig. 2B). Furthermore, no neutrophils were detectable in the BALF of MyD88-/- mice upon LPS administration, in contrast to the dose-dependent neutrophil recruitment seen in WT controls (Fig. 2C). No neutrophil recruitment was observed in lung and BAL of either mouse group after intranasal NaCl (Fig. 2C). Finally, vascular leak was assessed by the measurement of protein levels in the BALF. Protein levels were increased in WT mice, but not in MyD88-/- mice 24 h after LPS administration (Fig. 2D). To test a contribution of the MyD88-independent signaling, we tested TRIF-deficient mice (TRIF-/-) in the same conditions. TRIF-/- had normal MPO activity, neutrophil recruitment in the BALF, and vascular leak in response to aerogenic LPS (data not shown). Therefore, MyD88 signaling is critical for LPS-induced acute neutrophil sequestration into the lung parenchyma and the bronchoalveolar space, and for the microvascular damage resulting in fluid leak.

LPS-induced TNF, IL-12 p40, and KC in the BALF is MyD88-dependent

Because MyD88 is critical to signal some LPS-induced cytokine responses in murine bone marrow-derived macrophages in vitro (36), we asked whether absence of MyD88 prevents the production of cytokines in the BALF after intranasal LPS administration. Increased levels of TNF at 3 h and IL-12 p40 at 24 h after LPS are found in the BALF of WT mice that are significantly lower in MyD88-/- mice (Fig. 3, A and B). Unlike systemic LPS treatment, the intranasal administration of LPS failed to increase TNF and IL-12 p40 levels in serum of WT mice (data not shown). Furthermore, we extended our analysis of the inflammatory response in BALF by testing the production of the chemokine KC, which is critical for neutrophil recruitment. We showed that KC is up-regulated by LPS in WT mice, but undetectable in the BALF of MyD88-/- mice (Fig. 3C).

Therefore, the production of TNF, IL-12 p40, and of the chemokine KC upon aerogenic LPS administration is largely MyD88-dependent.

TLR4-MyD88-dependent, but IL-1R1- and caspase-1-independent, bronchoconstriction and neutrophil recruitment

Although MyD88 is a common adaptor of TLRs, it is also involved in the signaling of IL-1R and IL-18R. To exclude that the observed effects in MyD88-/- mice were due to defective IL-1 signaling, we investigated the response to aerogenic LPS of IL-1R1-/- mice. IL-1R1-/- mice, unlike MyD88-/- mice, responded with bronchoconstriction and an intense neutrophil recruitment to intranasal LPS (Fig. 4, A and B). Moreover, bronchoconstriction and neutrophil recruitment were also observed in caspase-1 (ICE-)/- mice (29), which do not convert their precursors into active IL-1β and IL-18 (Fig. 4, A and B). The difference in intensity of the responses to airway LPS observed between IL-1R1-/-, ICE-/-, and WT C57BL/6 mice may be attributed in part to variation in mouse strains sensitivity to LPS. Indeed, ICE-/- and IL-1R1-/- mice are from 129 genetic background backcrossed 7-fold onto C57BL/6, two background with marked differences in bronchoconstriction, neutrophil recruitment, and TNF and MIP-2 secretion in response to inhaled LPS or to involvement of others genes than TLR4 and MyD88 in the modulation of lung response to LPS (37). In contrast, TLR4-deficient mice experienced neither bronchoconstriction nor neutrophil recruitment in the BALF after aerogenic LPS administration (Fig. 4, C and D). These data demonstrate that IL-1R and IL-18R are dispensable for bronchoconstriction and neutrophil recruitment upon aerogenic LPS, and show that TLR4 signaling, but neither IL-1βR nor IL-18R signaling, is critical in the MyD88-mediated, acute pulmonary inflammatory response to LPS.

Establishment of chimeric mice

The data above suggested that TLR4-mediated signaling through MyD88 is required for LPS-induced acute respiratory distress response. Because TLRs are expressed on a broad range of tissues and cells, we next addressed the respective role of bone marrow-derived and lung resident cells for sensing LPS and for the induction of the TLR4-MyD88-dependent activation signal. MyD88-/- and WT mice were lethally irradiated and reconstituted with either WT or MyD88-/- bone marrow cells. The resulting WT→knockout (KO) and KO→WT chimeric mice were tested for reconstitution of hemopoesis with donor cells and for pulmonary
alveolar macrophages repopulation, 3 mo postirradiation. The chimerism of the mice was first established using GFP transgenic mice as donors or recipients (31). Repopulation of blood and lungs by donor cells was controlled 3 mo posttransplantation. We showed that 3 mo after lethal irradiation of MyD88\(^{-/-}\) mice and their reconstitution with GFPMyD88\(^{+/+}\) bone marrow cells (GFPKO\(\rightarrow\)WT), the circulating blood CD11b\(^{+}\) mononuclear cells were of donor origin (74.5%) as compared with the maximal value of 81% circulating GFP\(^{+}\) cells in GFP transgenic mice (Fig. 5A). Conversely, after lethal irradiation of MyD88\(^{-/-}\) mice and reconstitution with bone marrow cells from MyD88\(^{+/+}\) mice transgenic for GFP (GFPWT\(\rightarrow\)KO), the circulating blood monocytes were mostly of donor origin (67.3% circulating GFP\(^{+}\) cells; Fig. 5B). More importantly, alveolar macrophages recovered in the BALF of GFPKO\(\rightarrow\)WT mice or GFPWT\(\rightarrow\)KO mice were also mainly of donor origin (83.7% and 60% GFP\(^{+}\) cells, respectively, in comparison to 97.8% GFP\(^{+}\) cells in BALF from GFP transgenic mice; Fig. 5, C and D). These results indicate that repopulation of bronchoalveolar space with donor macrophages is effective although not complete, as expected, 3 mo after reconstitution. In addition, cells recovered in the BALF of KO\(\rightarrow\)GFPWT mice 24 h after intranasal administration of LPS were also of donor origin (97.1%...
GFP− cells), indicating that MyD88−/− neutrophils are normally recruited into the alveolar space (data not shown).

Then the function of the reconstituting cells was assayed for all chimera. Peripheral blood cells were tested for TNF production after LPS stimulation in vitro. Fig. 5E shows that blood cells from WT→KO had a normal TNF production as compared with MyD88−/− (WT) mice, while leukocytes derived from KO→WT mice produced only very low levels of TNF, similar to MyD88−/− (KO) leukocytes. The same results were obtained when BMDM from WT→KO and KO→WT chimeric mice were tested for TNF production after LPS or LTA stimulation in vitro (Fig. 5F). These data demonstrate the efficiency of the reconstitution protocol for creating functional MyD88 chimera.

Critical role of resident cells for LPS-induced bronchoconstriction

We first investigated LPS-induced bronchoconstriction and asked whether the unresponsiveness of MyD88−/− mice could be corrected by bone marrow transplantation. Most interestingly, LPS did not induce bronchoconstriction in irradiated MyD88−/− mice reconstituted with WT bone marrow (WT→KO; Fig. 6A) similar to what was shown in naive MyD88−/− mice (Fig. 1A). Because

FIGURE 3. MyD88-dependent, LPS-induced TNF, IL-12 p40, and KC production in BALF. LPS was given at 50 μg by the intranasal route to MyD88−/− (■) and to WT (□) mice, and the BALF was analyzed at 24 h for TNF (A), for IL-12 p40 (B), and KC (C) concentration at 24 h by ELISA. The data represent mean values ± SD from three independent experiments (n = 4 mice per group; *, p < 0.05).

FIGURE 4. Bronchoconstriction and neutrophil recruitment in the absence of IL-1R1, caspase-1, or TLR4. MyD88−/−, IL-1R1−/−, caspase-1 (ICE)−/−, and WT mice received LPS at 50 μg by the intranasal route. Bronchoconstriction was analyzed by PenH levels for 3 h (A), and neutrophils in BALF were enumerated at 24 h (B); the airway response is expressed as mean ± SEM of PenH (**, p < 0.001 for MyD88−/− vs ICE−/−, MyD88−/− vs IL-1R1−/−, and WT vs ICE−/−; *, p < 0.01 for WT vs IL-1R1−/− and WT vs MyD88−/−; n = 4 mice per group). Neutrophil accumulation is expressed as mean values ± SD (n = 4 mice per group; *, p < 0.05). TLR4−/− and WT mice received LPS at 10 μg by the intranasal route, bronchoconstriction was analyzed for 3 h (C); the airway response is expressed as mean ± SEM of PenH (*, p < 0.01 from 90 to 360 min, n = 4 mice per group); neutrophil accumulation was counted in BALF at 24 h (D) and is expressed as mean values ± SD (n = 4 mice per group; *, p < 0.05).
we controlled that 60% of alveolar macrophages are from donor origin in this type of chimera, alveolar macrophages seem inefficient in mediating bronchoconstriction. Conversely, WT mice reconstituted with MyD88-deficient bone marrow (KO→WT) showed a strong bronchoconstrictive response (Fig. 6A) similar to that seen in WT controls (Fig. 1A). Moreover, LPS-induced bronchoconstriction in irradiated MyD88−/− mice reconstituted with WT bone marrow (WT→WT) and irradiated MyD88−/− mice reconstituted with deficient bone marrow (KO→KO) were equivalent to KO→WT and WT→KO chimeric mice, respectively, as represented by AUC (Fig. 6B). Thus, LPS-induced bronchoconstriction is determined by the genotype of the recipient, irrespective of the donor cells used for hemopoietic reconstitution. These results suggest that MyD88 signaling by radiosensitive resident cells is critical for LPS-induced bronchoconstriction.

**Critical role of hemopoietic cells for LPS-induced cytokine production**

Then we measured cytokine production in the BALF from chimeric mice. TNF and IL-12 p40 production were fully restored by WT bone marrow in MyD88−/− mice (WT→KO; Fig. 6, C and D). In contrast, MyD88−/− bone marrow cell reconstitution abrogated TNF and IL-12p40 secretion by WT mice in response to LPS. Thus, TNF and IL-12p40 secretion into the bronchoalveolar space is fully dependent on MyD88 signaling from cells of hemopoietic origin.

**Resident cells and hemopoietic cells are required for LPS-induced inflammation**

Finally, we tested the neutrophil recruitment in the chimeric mice. WT bone marrow reconstitution of MyD88−/− (WT→KO) restored neutrophil recruitment to the BAL after LPS at 50 μg (4.8×10^5 neutrophils/BAL; Fig. 6E) in comparison to the absence of neutrophil recruitment in MyD88−/− mice (Fig. 2C). Nevertheless, the neutrophil recruitment observed in WT→KO chimeric mice is modest as compared with the recruitment observed in WT mice (35×10^5 neutrophils per BAL; Fig. 2C). In contrast, reconstitution of WT mice with MyD88−/− bone marrow (KO→WT) reduced the neutrophil recruitment observed in WT mice from 35 to 6×10^5 neutrophils per BAL (Fig. 6E). Therefore, the genotype of the donor bone marrow cells seems to influence only in part the neutrophil recruitment, and both resident and myeloid cells are required for a full recruitment of neutrophils via MyD88 signaling.

Altogether, the results from MyD88 bone marrow chimera suggest that LPS-induced bronchoconstriction is mediated via MyD88 by a radiosensitive, nonhemopoietic cell type, while TNF and IL-12p40 production depends on myeloid cells from bone marrow origin. Efficient neutrophil recruitment depends upon TLR4-MyD88 signals mediated through both resident and myeloid cells. Therefore, our data separate, for the first time, the essential role of LPS-TLR-MyD88 pathway in myeloid cells involved in proinflammatory cytokine synthesis from that of resident lung cells leading to bronchoconstriction.

**FIGURE 5.** Reconstitution of blood and BAL cells after bone marrow transfer. Bone marrow chimeras were prepared by lethal irradiation of WT and MyD88−/− (KO) mice and reconstitution with either source of unseparated bone marrow cells (2×10^6 cells per mouse). Hemopoietic reconstitution was monitored after 3 mo by flow cytometry analysis of GFP fluorescent cells (A–D). A. Flow cytometry of peripheral blood from WT mice irradiated and reconstituted with GFP transgenic (GFP) KO bone marrow cells compared with peripheral blood of naive GFP and WT mice. B. Flow cytometry of peripheral blood from KO mice irradiated and reconstituted with GFP WT bone marrow cells compared with peripheral blood of naive GFP and WT mice. C. Flow cytometry of alveolar macrophages from WT mice irradiated and reconstituted with GFP KO bone marrow cells compared with alveolar macrophages from GFP and WT mice. D. Flow cytometry of alveolar macrophages from KO mice irradiated and reconstituted with GFP WT bone marrow cells compared with alveolar macrophages from GFP and WT mice. E. In vitro LPS-induced TNF secretion by blood cells depends on the genotype of the hemopoietic cells used for bone marrow transplantation. Blood was obtained 3 mo postreconstitution: WT mice were irradiated and reconstituted with KO bone marrow cells (KO→WT), and KO mice were irradiated and reconstituted with WT bone marrow cells (WT→KO). F. In vitro LPS-induced TNF secretion by BMDM depends on the genotype of the hemopoietic cells used for bone marrow transplantation. BMDM were obtained 3 mo postreconstitution; WT mice were irradiated and reconstituted with KO bone marrow cells (KO→WT), and KO mice were reconstituted with WT bone marrow cells (WT→KO). BMDM were incubated with LPS (0.1 μg/ml) or LTA (10 μg/ml), and TNF was measured in the supernatant after 24 h. Data are from one experiment representative of two independent experiments (n = 6; *, p < 0.05; **, p < 0.01).
FIGURE 6. MyD88-dependent LPS-induced bronchoconstriction is mediated by resident cells, unlike the proinflammatory cytokine response. Bone marrow chimeras were prepared by lethal irradiation of either WT and MyD88−/− mice, followed by bone marrow reconstitution as described in Fig. 5. A, Bronchoconstriction (PenH) was recorded immediately after LPS (50 μg) intranasal administration (p < 0.001) from 90 to 360 min. B, AUC measured from 60 to 180 min. C and D, BALF was analyzed at 24 h for TNF (C) and IL-12 p40 levels (D) by ELISA. E, Neutrophil recruitment into the BAL at 24 h. The data represent mean values ± SD from one experiment representative of three independent experiments (n = 4, **, p < 0.01; n.d., not detected).

Discussion

Lung inflammation in response to LPS is essential for host defense to infection, but is deleterious after repeated exposure due to high concentrations of LPS in organic dust, air pollution in the environment, and it contributes to the development and progression of asthma and other airway diseases. In vitro studies have shown that LPS signals via TLR4 and MyD88 to activate the transcription factor NF-κB (9). In lungs, which constitute a very compartmentalized organ with continuous exposure to environmental Ags, the transduction pathways and the cell types implicated in the response to LPS are not fully explored. A recent report pointed to a site-specificity of the innate lung responses showing that pulmonary APCs differ from APCs at other sites in their capacities to engage MyD88-dependent and MyD88-independent (TRIF-dependent) pathways in response to specific TLR engagement (27). Here, we explored the two described pathways of the TLR4-dependent response to LPS and show in the local LPS-induced acute pulmonary inflammation model that bronchoconstriction; TNF, IL-12p40, and KC secretion; vascular leak; and neutrophil recruitment into the lung and the bronchoalveolar space are strictly dependent on the adaptor protein MyD88. By contrast, bronchoconstriction and inflammatory response are independent of the adaptor protein TRIF. Because MyD88 is involved not only in TLR, but also in IL-1R and IL-18R signaling, we investigated IL-1R1−/− mice, which cannot respond to IL-1β, and caspase-1 (ICE)−/− mice, which cannot convert their precursors into active IL-1β and IL-18. We show that IL-1R1 and caspase-1 are dispensable for LPS-induced bronchoconstriction and neutrophil recruitment. In fact, IL-1β was reported to play a protective role on airway smooth muscle (38–41). Moreover, we show here that TLR4−/− mice do not develop bronchoconstriction and neutrophil inflammation in response to local LPS. The involvement of TLR4 signaling in neutrophil recruitment in response to aerosol LPS was shown by others in C3H/HeJ endotoxin resistant mice bearing a point mutation of the tlr4 gene and in C57BL/10ScNcr mice with a complete deletion of the tlr4 gene (37) and recently in TLR4-deficient mice (42–44). In humans, mutations affecting the extracellular domain of the TLR4 receptor were shown to be associated with blunted response to inhaled LPS (6, 7). However, the role of TLR4-signaling in direct LPS-induced bronchoconstriction was not investigated before in mice. Hollingsworth et al. (42, 43) studied airway hyperresponsiveness to methacholine, mediated by different mechanisms to LPS-induced bronchoconstriction. Together, our data indicate that the MyD88-dependent pathway is essential for LPS-induced lung inflammation and that this signaling is mediated by TLR4, rather than IL-1βR or IL-18R. Indeed, impairment of IL-1R or IL-1 plus IL-18 cleavage by ICE does not prevent bronchoconstriction or neutrophil recruitment in the airways. However, we cannot exclude that other MyD88-dependent pathways such as the recently described involvement of MyD88 in cross-talk with the focal adhesion kinase (45) or other members of the IL-1R family may be involved (46).

We then asked whether the defect observed in MyD88−/− mice could be corrected by bone marrow transplantation. We demonstrate here that the absence of LPS-induced bronchoconstriction observed in MyD88−/− mice could not be corrected by WT bone marrow cells, nor could the nonresponsive MyD88−/− phenotype be transferred into WT mice upon hematopoietic reconstitution. These results were unexpected and indicated that TLR sensing by radioresistant resident cells in the lung may be critical and sufficient to provoke bronchoconstriction to aerogenic LPS. Potential cell populations sensing LPS in a MyD88-dependent fashion and contributing to bronchoconstriction include epithelial, endothelial, smooth muscle, or stromal cells. As a primary interface between inhaled agents and lung, epithelial cells lining the mammalian airways are likely candidates for LPS sensing. Epithelial cells provide both barrier and signaling functions to protect against infections. Indeed, the airway epithelium has been shown to express TLRs
and distinct pulmonary epithelial cell from humans and mice were shown to respond to LPS through TLR4-MyD88 signaling despite the intracellular localization of TLR4 (26). This particular localization of TLR4, expected to prevent the development of chronic inflammation, underlines the specificity of lungs that are continuously exposed to microbial challenges. Recent studies pointed toward a prominent role of airway epithelial cells in orchestrating the airway inflammatory response to LPS, showing that selective inhibition of NF-κB in epithelial cell reduced neutrophil influx and secretion of MIP-2 and TNF into the airways (50–52). Upon activation, epithelial cells express inflammatory cytokines such as TNF and IL-1β and cytokine receptors including TNF-R1, which are also expressed on vascular smooth muscle cells (52, 53). Also TNF and IL-1β were shown to regulate pulmonary smooth muscle contraction via epithelium-dependent mechanism such as the release of epithelium-derived factors (38–40). These data are in accordance with the interpretation of our results that LPS-induced bronchoconstriction depends on MyD88-signaling in epithelial cells although the possible involvement of endothelial cells cannot be excluded (11).

We show that WT bone marrow-derived cells were able to restore TNF and IL-12 p40 secretion in the BALF and, conversely, hemopoietic reconstitution with MyD88-deficient bone marrow cells transferred the MyD88−/− phenotype, indicating that bone marrow-derived cells are critically implicated in cytokine secretion in the bronchoalveolar space. Alveolar macrophages appear to be the main source of TNF and IL-12p40 production in the air space in response to LPS via MyD88 signaling. Indeed, alveolar macrophages were shown to produce TNF and other cytokines in the BALF in response to inhaled LPS (54).

WT bone marrow-derived cells restored partial neutrophil recruitment to air space of MyD88−/− mice, and MyD88−/− bone marrow only partially reduced neutrophil recruitment in WT mice, indicating that both resident cells and bone marrow-derived cells contribute to neutrophil recruitment. This recruitment in KO → WT mice could not be due to MyD88+/− alveolar macrophages as we verified efficient repopulation of the lungs with donor macrophages 3 mo after irradiation. Adequate repopulation of the lungs with donor macrophages was shown to occur 60 days after bone marrow transplantation, well after complete repopulation of peripheral blood has occurred (55). Moreover, absence of detectable TNF in BAL of KO → WT is an indicator of reduced WT recipient cells and effective repopulation by MyD88−/− donor macrophages. Hollingsworth et al. (43) reported a smaller neutrophil recruitment with TLR4KO → WT chimera than with WT → TLR4KO chimera and concluded on an effect of macrophage-derived factors on airway epithelium or endothelium independent of tlr4 expression rather than a role of resident cells. In innate resistance to uropathogenic E. coli, infection has been shown to be TLR4 dependent using a bone marrow transplant approach, and TLR4 expression on both hemopoietic and stromal/epithelial cells were required (56). Indeed, several reports point to the important role of epithelial cells in neutrophil recruitment (50–52). Based on our results, together with the absence of detectable TNF in the BAL of KO → WT mice, we show that TNF from alveolar macrophages is not the sole mediator implicated in neutrophil recruitment. Other cytokines or chemokines produced by different cell types like epithelial cells and alveolar macrophages seem to be involved. Airway epithelial cells and alveolar macrophages were claimed as the first line of defense in expressing chemokines and cytokines to direct influx and activation of inflammatory cells, but their role in bronchoconstriction was rarely reported. Our data show that bronchoconstriction and neutrophil inflammation in response to LPS are independent events. Indeed, we have observed in TNF-deficient mice that bronchoconstriction is absent despite normal neutrophil recruitment in response to inhaled LPS (S. Schnyder-Candrian, B. Schnyder, N. Noulia, and I. Couillin, unpublished observations).

In conclusion, our data demonstrate a central role of the MyD88 adaptor molecule for bronchoconstriction, proinflammatory cytokine secretion, vascular leak, and neutrophil recruitment in response to airway LPS, which are absent in MyD88-deficient mice, pointing to the critical role of this central adaptor molecule in innate host defense and inflammation in lung. By contrast, the TRIF adaptor molecule is dispensable for the LPS-induced acute pulmonary inflammation. Furthermore, for the first time, we clearly separate bronchoconstriction from air space proinflammatory cytokine secretion in response to aerogenic LPS administration. Although both require MyD88-mediated signaling, our data clearly show that these two independent events are mediated by different cell populations. The bone marrow chimera demonstrate that LPS-induced bronchoconstriction is mediated by radiotransient resident cells, likely epithelial cells, while TNF and IL-12p40 production depends on MyD88 pathway in the bone-marrow-derived myeloid cells. Efficient neutrophil recruitment requires MyD88 signaling from both resident and myeloid cells and was independent of bronchoconstriction. Therefore, the data support the notion that nonmyeloid cells such as epithelial cells are critical in response to aerogenic LPS.

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Disclosures
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References


