Nonredundant roles of TIRAP and MyD88 in airway response to endotoxin, independent of TRIF, IL-1 and IL-18 pathways

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Inhaled endotoxins induce an acute inflammatory response in the airways mediated through Toll-like receptor 4 (TLR4) and myeloid differentiation factor 88 (MyD88). However, the relative roles of the TLR4 adaptor proteins TIRAP and TRIF and of the MyD88-dependent IL-1 and IL-18 receptor pathways in this response are unclear. Here, we demonstrate that endotoxin-induced acute bronchoconstriction, vascular damage resulting in protein leak, Th1 cytokine and chemokine secretion and neutrophil recruitment in the airways are abrogated in mice deficient for either TIRAP or MyD88, but not in TRIF deficient mice. The contribution of other TLR-independent, MyD88-dependent signaling pathways was investigated in IL-1R1, IL-18R and caspase-1 (ICE)-deficient mice, which displayed normal airway responses to endotoxin. In conclusion, the TLR4-mediated, bronchoconstriction and acute inflammatory lung pathology to inhaled endotoxin critically depend on the expression of both adaptor proteins, TIRAP and MyD88, suggesting cooperative roles, while TRIF, IL-1R1, IL-18R signaling pathways are dispensable.

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Inflammation of the airways due to environmental agents including endotoxin or lipopolysaccharide (LPS) plays an important role in the development and progression of chronic respiratory disease including asthma.^{1–5} Pathologic changes induced by endotoxin inhalation include acute respiratory distress syndrome, neutrophil recruitment, injury of the alveolar epithelium and endothelium with protein leak in the alveolar space.^{6,7} In mice, aerogenic exposure to endotoxin from Gram-negative bacteria induces acute pulmonary inflammation, local TNF production, alveolar-capillary leak and also a direct bronchoconstriction.^{8,9} Toll-like receptor (TLR) 4 and CD14 play a critical role in the pulmonary response to systemic endotoxin administration^{10–12} and we showed recently that TLR4

expression level determines the extent of acute pulmonary response to inhaled endotoxin.¹³ Aero-genic endotoxin exposure also induces neutrophil recruitment into the alveolar space,⁸ which is MAPK-dependent, but independent of the secretion of TNF.⁹

TLR4 uses different combinations of TIR domain containing adaptor proteins to activate distinct signaling pathways, most prominently MyD88 and TIR domain containing adaptor inducing interferon-beta (TRIF), leading to the production of proinflammatory cytokines and type I IFNs, respectively.^{14–17} Absence of MyD88 confers resistance to systemic endotoxin-induced shock.¹⁸ TIRAP-deficient mice are also resistant to the toxic effects of LPS,¹⁹ with defective induction of TNF, IL-6 or IL-12p40 and delayed activation of NF- κ B and MAP kinases^{20,21} Indeed, MyD88 and TIRAP are involved in early activation of NF- κ B and MAP kinases^{19,21–23} whereas TRIF and TRAM are critical for late activation of NF- κ B as well as IRF-3 activation.^{24,25}

The inflammatory responses in lung, a site of continuous exposure to environmental antigens, are believed to be different from those present in less

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exposed, accessible sites.^{26,27} We showed recently that the TLR adaptor MyD88 is critical for the airway inflammatory response to endotoxins.²⁸ MyD88 is at the crossroad of multiple TLR-dependent and TLR-independent signaling pathways, including IL-1R and IL-18R, or FAK.²⁹ In certain infection models the extreme sensitivity of MyD88-deficient mice may be ascribed, at least in part, to deficient IL-1R/IL-18R signaling pathways, as shown recently for cutaneous *Staphylococcus aureus* infection³⁰ and our own unpublished results on mycobacterial infections (BR, VQ, DT, VV). Here, we investigated the underlying mechanisms of the MyD88-dependent airway inflammatory response. Using genetically modified mice we analyzed the relative contribution of the TLR4 adaptor proteins TIRAP, MyD88 and TRIF as well as the contribution of the MyD88-dependent, TLR-independent IL-1R and IL-18R signaling pathways on inhaled endotoxin induced lung injury.

TIRAP has recently been shown to be essential for LPS-induced lung inflammation.³¹ Here, we extend this observation to LPS induced bronchoconstriction and present a side-by-side evaluation of the several TLR4- and/or MyD88-dependent pathways potentially involved in these responses. We demonstrate that TIRAP together with MyD88 are both essential and nonredundant for LPS-TLR4-induced acute pulmonary inflammation response, while other signals contributing to TLR4- or MyD88-dependent pathways such as TRIF, IL-1R and IL-18R signals are dispensable. LPS-induced bronchoconstriction, pulmonary neutrophil sequestration, vascular leak, TNF, IL-12p40 and KC secretions were abrogated in either TIRAP- or MyD88-deficient mice while these parameters were unaffected in mice deficient for TRIF, IL-1R, IL-18R or ICE.

Materials and methods

Mice

TIRAP^{-/-},²¹ MyD88^{-/-},¹⁸ IL-1R1^{-/-},³² IL-18R^{-/-},³³ caspase-1^{-/-},³⁴ TLR4^{-/-},³⁵ and *Trif*^{Lps2} homozygote mutant mice¹⁵ backcrossed 10 times for MyD88^{-/-} and TLR4^{-/-}, and seven times for IL-1R1^{-/-} and caspase-1^{-/-}, on the C57BL/6 genetic background and wild-type control C57BL/6 (WT) were bred in our animal facility at the Transgenose Institute (CNRS, Orleans). For experiments, adult (6–10 weeks old) mice 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

Endotoxin (10 µg) from *Escherichia coli* (serotype 055: B5, Sigma, St Louis, MO, USA) or saline were

given by the aerogenic route using nasal instillation in a volume of 50 µl under light i.v. ketamine-xylazine anesthesia. The airway resistance was evaluated by whole-body plethysmography⁹ at several time points. Unrestrained conscious mice were placed in whole-body plethysmography chambers (EMKA Technologies, France). Enhanced Respiratory Pause (PenH) measurements were collected 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 $\text{PenH} = (\text{Te}/\text{RT} - 1) \times \text{PEF}/\text{PIF}$, where Te is expiratory time, RT is relaxation time, PEF is peak expiratory flow, and PIF is peak inspiratory flow.³⁶

Bronchoalveolar Lavage Fluid

Bronchoalveolar Lavage Fluid (BALF) was collected 3 or 24 h after endotoxin administration by cannulating the trachea under deep i.p. ketamine-xylazine anesthesia and washing the lung four times with 0.5 ml of saline at room temperature as described.³⁶ The lavage fluid was centrifuged 10 min at 2000 r.p.m. 4°C and the supernatant frozen for cytokine content. The cell pellet was resuspended in PBS, counted in a hemacytometer chamber and cytospin preparations were made using a Shandon cytocentrifuge (1000 r.p.m., 10 min). The cells were stained with Diff-Quick (Dade Behring, Marburg, Germany). The supernatant was used for the measurement of cytokine and protein levels. TNF, IL-12 p40 and KC were measured by enzyme-linked immunosorbent assay (R&D Duoset, Minneapolis, MO, USA). For protein determination, Bradford stain was added to the supernatant as described by the manufacturer (Bio-Rad, Ivry sur Seine, France) using an ovalbumin standard and absorbance was measured at 595 nm (Uvikon spectrophotometer, Kontron Zurich, Switzerland).

Microscopy and Myeloperoxidase Activity in Lung

After bronchoalveolar lavage and lung perfusion, the mice were sacrificed. The lung were fixed in 4% buffered formaldehyde for standard microscopic analysis, 3 µm sections were stained with hematoxylin and eosin (H&E) as described previously.³⁶ Neutrophil and erythrocyte accumulation in alveoli, disruption of alveolar septae and activation of alveolar epithelial cells were quantified using a semiquantitative score with increasing severity of changes (0–5) by two independent observers including a trained pathologist (BR). Groups of five to eight mice per genotype and 10 randomly selected high-power fields ($\times 400$ magnification) per animal were analyzed.

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, centrifuged and the supernatant was discarded. The pellets were resuspended in 1 ml PBS containing 0.5% hexadecyltrimethyl ammonium bromide (HTAB) and 5 mM ethylene-diamine tetra-acetic acid (EDTA). Following centrifugation, 50 μ l of supernatants were placed in test tubes with 200 μ l PBS-HTAB-EDTA, 2 ml Hanks' balanced salt solution (HBSS), 100 μ l of *o*-dianisidine dihydrochloride (1.25 mg/ml), and 100 μ l H₂O₂ 0.05%. After 15 min of incubation at 37°C in an agitator, the reaction was stopped with 100 μ l NaN₃ 1%. The MPO activity was determined as absorbance at 460 nm against medium.

BMDM Culture and Stimulation *In Vitro*

Primary bone marrow-derived macrophages (BMDM) were obtained from femoral bone marrow as described.³⁷ In brief, cells from the femur were isolated and cultured at 10⁶ cells/ml for 7 days in Dulbecco's minimal essential medium (DMEM, Sigma) supplemented with 20% horse serum and 30% L929 cell conditioned medium as a source of M-CSF. At 3 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 10⁵ cells/well) and stimulated with LPS (*E. coli*, serotype 055: B5, at 100 ng/ml). Cell supernatants were harvested after 18–24 h of stimulation for TNF, IL-12p40 and KC measurement: analyzed immediately or stored at –20°C.

Statistical Analysis

Statistical evaluation of differences between the experimental groups was determined by Mann–Witney 'U' test for plethysmography experiments,

and Student's *t*-test for others data, using Prism software. *P*-values of <0.05 were considered statistically significant.

Results

Endotoxin-Induced Bronchoconstriction Depends on TIRAP and MyD88, not TRIF

Intranasal administration of endotoxin (10 μ g LPS) induced direct bronchoconstriction in C57BL/6 wild-type mice within 90 min, whereas TIRAP^{–/–} mice were completely unresponsive. LPS-induced bronchoconstriction increased for the next hours with a maximum enhanced pause (PenH) around 150 min and then decreased towards basal level after 4 h (Figure 1a). Bronchoconstriction was absent in TIRAP^{–/–} mice (Figure 1a) and the difference to wild-type mice as represented by the area under the curve (AUC) was highly significant (Figure 1b). Similarly, MyD88^{–/–} and TLR4^{–/–} mice were also unresponsive to endotoxin, while *Trif*^{fl^{ps2}} homozygote mutant mice functionally deficient for TRIF had normal bronchoconstriction response to aerogenic endotoxin (Figure 1c). Therefore, both adaptor proteins TIRAP and MyD88 play a critical and non-redundant role in the TLR4-dependent response for acute bronchoconstriction induced by intranasal instillation of LPS, while TRIF signaling is not required.

Neutrophil Recruitment into the Lung upon Endotoxin Challenge Depends on TIRAP and MyD88, but not TRIF Signaling

We then asked whether the absence of bronchoconstriction in TIRAP^{–/–} mice is associated with a reduced neutrophil recruitment into the lung. No neutrophils were detected in the BAL fluid of

Figure 1 Absence of LPS-induced bronchoconstriction in mice deficient for TIRAP or MyD88, not TRIF. **(a)** Bronchoconstriction in wild-type control mice, but not in TIRAP^{–/–} mice in response to aerogenic endotoxin (10 μ g LPS given by the i.n. route). The airways response is expressed as mean $\pm$ s.e.m. of PenH ($P < 0.01$ from 90 to 360 min). **(b)** Area under the curve (AUC) measured from 60 to 180 min after endotoxin in TIRAP^{–/–} and wild-type mice. **(c)** Absence of bronchoconstriction (AUC) in MyD88 and TLR4^{–/–} mice, but not in TRIF^{–/–} mice. Data are from one experiment representative of two independent experiments ($n = 8$ mice per group; ** $P < 0.01$; ns, not significant).

TIRAP^{-/-} mice and MyD88^{-/-} mice at 24 h upon intranasal endotoxin administration, in contrast to wild-type controls which had high neutrophil counts in the BAL (Figure 2a). In order to quantify neutrophil recruitment in the lung, myeloperoxidase (MPO) activity was determined in lung tissue homogenates. While wild-type mice showed a significant MPO activity 24 h after LPS challenge, TIRAP^{-/-} and MyD88^{-/-} mice had essentially no MPO activity in lungs (Figure 2b), similar to untreated controls. The recruitment of neutrophils was not merely delayed in TIRAP^{-/-} mice as MPO activity did not increase at 24 h and 72 h (data not shown). Finally, endothelial damage was assessed by the measurement of protein leak into the BAL fluid. Protein levels were increased in wild-type mice, but not in TIRAP^{-/-} and in MyD88^{-/-} mice (Figure 2c) 24 h after endotoxin administration.

To assess the contribution of the MyD88-independent signaling, we tested TRIF deficient mice in the same conditions. *Trif*^{ps2} homozygote mutant mice had essentially normal neutrophil recruitment, lung MPO activity and protein levels in the BAL fluid in response to aerogenic endotoxin 3 h after endotoxin administration (Figure 2d–f). Therefore, TIRAP-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 protein leak, while TRIF signaling is dispensable.

TIRAP and MyD88 are Essential for TNF, IL-12 p40 and KC Secretion in the Airways

MyD88 is critical to signal some LPS-induced Th1 cytokine responses in murine BMDM *in vitro*.³⁸ We show that the production of TNF, IL-12p40 and NO by LPS activated BMDM is also TIRAP-dependent, as TIRAP^{-/-} macrophages are unresponsive to LPS *in vitro* (Figure 3a). We then quantified the production of cytokines and chemokines in BAL fluid and lung homogenates after intranasal endotoxin administration. Increased levels of TNF and KC at 3 h and IL-12 p40 at 24 h after endotoxin challenge were found in the BAL fluid of wild-type mice which were significantly reduced in TIRAP^{-/-} mice (Figure 3b), reminiscent of the results recently reported in MyD88^{-/-} mice.²⁸ Pulmonary TNF, IL12 p40 and KC production in response to local endotoxin was also severely impaired in TIRAP^{-/-} mice (Figure 3c). Therefore, both TIRAP and MyD88

Figure 2 Reduced neutrophil recruitment and vascular leakage in the lung of LPS-challenged TIRAP^{-/-} and MyD88^{-/-} mice. (a) Neutrophil counts in the BALF were assessed 24 h after LPS administration (10 μ g, i.n.) in wild-type (TIRAP^{+/+}), TIRAP^{-/-} and MyD88^{-/-} mice. (b) Pulmonary MPO activity was measured in order to quantify the neutrophils in the lung tissue 24 h after endotoxin challenge. (c) Total protein levels were measured in the BALF 24 h after endotoxin challenge. (d) Neutrophil counts in the BALF were assessed 3 h after LPS administration (10 μ g, i.n.) in TRIF deficient mice. (e) Pulmonary MPO activity was measured 3 h after endotoxin challenge in lung of *Trif*^{ps2} homozygote mutant mice. (f) Total protein levels were measured in the BALF 3 h after endotoxin challenge. Data are representative of three independent experiments and are expressed as mean values $\pm$ s.d. ($n = 4$ mice per group; * $P < 0.05$; ** $P < 0.01$; nd, not detected; ns, not significant).

Figure 3 TIRAP-dependent airway TNF, IL-12p40 and KC production upon endotoxin challenge. (a) BMDM from TIRAP deficient and control mice were stimulated with endotoxin (100 ng/ml), or medium alone (control) and the supernatants assessed for TNF, IL-12p40 and KC production at 24 h by ELISA. (b) TNF, IL-12p40 and KC levels were determined by ELISA in the BAL fluid and (c) in lung homogenate from TIRAP^{-/-} and wild-type mice after LPS (10 μ g, i.n.; black bars) or saline (open bars) administration. The data represent mean values $\pm$ s.d. from three independent experiments ($n=4$ mice per group; * $P<0.05$; ** $P<0.01$).

adaptors are essential for the synthesis of TNF, IL-12 p40 and of the chemokine KC upon aerogenic endotoxin administration.

Endotoxin-Induced Neutrophil Recruitment and Lung Injury is TIRAP and MyD88 Dependent

Finally, we asked whether the neutrophil recruitment in the capillary and alveolar space and the damage of lung architecture seen upon endotoxin administration is TIRAP-dependent. The micrographs of the lung 24 h after endotoxin revealed a

complete absence of neutrophils in the capillaries and alveolar space in TIRAP^{-/-} mice (Figure 4a), similar to MyD88^{-/-} mice (Noulin *et al*²⁸ and data not shown). The lung damage reflected by epithelial cell activation and focal necrosis, disruption of alveolar septae and focal edema was found in wild-type mice, but absent in TIRAP^{-/-} (Figure 4a) and MyD88^{-/-} mice (data not shown). Neutrophil infiltration and alveolar damage were quantified on microscopic sections. Lungs from TIRAP^{-/-} and MyD88^{-/-} mice exhibited neither disruption of alveolar septae nor neutrophil in alveoli, while wild-type mice showed prominent neutrophil

recruitment into the alveolar space, epithelial cell activation and disruption of alveolar septae after endotoxin administration (Figure 4b–d).

Therefore, both TIRAP and MyD88 are pivotal to endotoxin-induced lung architecture damage and neutrophil recruitment.

Endotoxin-Induced Airway Response is Independent-of IL-1 and IL-18 Receptor Signaling

MyD88 is not only a common adaptor of most TLRs beside TLR3, but it is also involved in the signaling

of IL-1 and IL-18 receptors. The extreme sensitivity of MyD88 deficient mice in several infectious models turned out to be due to the absence of functional IL-1R/IL-18R signaling, shedding new light on the prominent role of IL-1 in some innate responses (Gamero and Oppenheim, 2006; our unpublished observations). Here, the similar effects observed in TIRAP and MyD88 deficient mice upon intranasal endotoxin challenge strongly suggest that the role of MyD88 in this condition is linked to its contribution to TLR4 signaling pathway. To address this point and rule out a contribution of MyD88 through IL-1 or IL-18 receptor signaling, we

Figure 4 TIRAP^{-/-} and MyD88-dependent inflammation and lung injury. The lungs of TIRAP^{-/-} mice were analyzed by microscopy 24 h after saline or LPS administration (10 μ g, i.n.). (a) Neutrophil infiltration, disruption of microarchitecture and activation alveolar epithelia observed in wild-type mice, were absent in TIRAP^{-/-} mice. Representative lung sections are shown at magnification $\times 100$. The lesions induced by endotoxins were assessed semiquantitatively (see Materials and methods): disruption of microarchitecture (b), activation of alveolar epithelial cells (c), neutrophils infiltration, (d) were essentially absent in TIRAP^{-/-} mice. Results are expressed as mean lesion score $\pm$ s.d. ($n \geq 5$ mice per group; * $P < 0.05$; ** $P < 0.01$).

investigated the response of IL-1R1 and IL-18R deficient mice to aerogenic endotoxin. Unlike MyD88^{-/-} mice, IL-1R1^{-/-} mice and IL-18R^{-/-} mice responded to intranasal endotoxin with bronchoconstriction and an intense neutrophil recruitment to the lung and the bronchoalveolar space (Figure 5a–c). Moreover, bronchoconstriction and neutrophil recruitment were also observed in caspase-1 (ICE) deficient mice,³⁴ which do not convert their precursors into active IL-1 β and IL-18 (Figure 5a–c). These data demonstrate that IL-1 and IL-18 receptors are dispensable for bronchoconstriction and neutrophil recruitment into the airways upon aerogenic LPS exposure. Therefore, TIRAP together with MyD88 is critical for TLR4 signaling resulting in acute pulmonary inflammatory response to local endotoxin, but neither IL-1 β nor IL-18 receptor signaling is essential for this response.

Discussion

It has been proposed that distinct combinations of adaptor molecules involved in the signaling platforms differ among TLRs and contribute to provide the observed specificity of the response induced by different TLR agonists.^{39,40} Endotoxin signals an inflammatory response by ligation to the CD14-TLR4 receptor complex recruiting several adaptor molecules and activating several kinases resulting in cell activation. TLR4 activation by endotoxin engages MyD88, TIRAP, TRIF and TRAM adaptor molecules, which induce differential activation programs in the cell.⁴¹ Here, we show that both TIRAP and MyD88 adaptor molecules are essential and nonredundant adaptors of TLR4 for endotoxin induced lung bronchoconstriction and acute airway inflammation while TRIF and the MyD88-dependent IL-1R and IL-18R pathways are dispensable for this response.

We recently demonstrated that MyD88 signaling is critical for endotoxin induced airway inflammation and that both MyD88 expressed in hemopoietic and resident cells are necessary for this response.²⁸ Several studies have shown that TIRAP is a critical component of the TLR4 signaling cascade to LPS in isolated cells¹⁹ but less is known about the role of TIRAP in lung immune response against LPS. A recent report documented the implication of TIRAP in the lung inflammatory response to LPS or live *E. coli* but no information was given on the LPS-induced bronchoconstrictive response.³¹ Further we documented some mechanistic differences in the induction of bronchoconstriction vs inflammatory cell recruitment upon LPS administration.⁹ Indeed, in TNF deficient mice bronchoconstriction was abrogated, but not the neutrophil sequestration in the lung. Thus LPS provokes acute bronchoconstriction which is TNF-dependent and p38 MAPK-mediated, while the neutrophil recruitment is independent of TNF although it also depends on LPS/TLR4-induced signals mediated by p38 MAPK.

Figure 5 Bronchoconstriction and neutrophil recruitment in the absence of IL-1R1, IL-18R or caspase 1. IL-1R1^{-/-}, IL-18R^{-/-}, caspase 1^{-/-} and wild-type mice received endotoxin at 10 μ g by the i.n. route, and bronchoconstriction was analyzed by PenH levels for 3 h (a). The airways response expressed as mean $\pm$ s.e.m. of AUC was normal in IL-1R1^{-/-}, IL-18R^{-/-}, caspase-1 (ICE)^{-/-} and wild-type mice ($P < 0.05$ from 110 to 360 min). The airway response was significantly higher in IL-1R^{-/-} mice ($P < 0.05$ from 90 to 360 min). (b) Neutrophil recruitment was assessed at 24 h in BALF and (c) in lung by MPO activity in lung homogenates. Data are representative of three independent experiments and are expressed as mean values $\pm$ s.d. ($n = 4$ mice per group; * $P < 0.05$). No significant difference in neutrophil recruitment was noted between the groups.

Here, we explored whether the adaptor TIRAP is required for the different aspects of TLR4-dependent airway response to endotoxin and investigated a possible contribution by TRIF signaling. We demonstrated that TIRAP is absolutely necessary to the LPS-induced MyD88-dependent inflammatory responses into the lung in mice and that MyD88 cannot

compensate the lack of TIRAP. We showed that both adaptor proteins TIRAP and MyD88 play a critical and nonredundant role in the TLR4-dependent response for acute bronchoconstriction, for neutrophil sequestration into the lung parenchyma and the bronchoalveolar space, for the microvascular damage resulting in protein leak and for the synthesis of TNF, IL-12 p40 and chemokine KC, upon aerogenic endotoxin, while TRIF signaling is not required. The role of TIRAP on direct LPS-induced bronchoconstriction was not investigated before. Therefore, TLR4 signaling pathway requires both MyD88 and TIRAP adaptors, which are non-redundant, for an *in vivo* endotoxin response leading to severe airway injury.

MyD88 is not only a common adaptor of TLRs, but it is also involved in the signaling of IL-1 and IL-18 receptors. The fact that similar effects were observed in mice deficient for either TIRAP or MyD88 upon intranasal endotoxin challenge strongly supported the notion that the implication of MyD88 in this response was due to its contribution to TLR4 signaling. To further establish this point we ruled out a contribution of MyD88 through either IL-1 or IL-18 signaling. Indeed, mice deficient for IL-1R1, IL-18R or caspase-1 that lack active IL-1 β and IL-18 maturation, had a normal response to aerogenic endotoxin, with bronchoconstriction and intense neutrophil recruitment to the lung parenchyma and bronchoalveolar space, much in contrast to the abrogated response seen in MyD88 or TIRAP deficient mice. Our data clearly established that IL-1 and IL-18 receptors are dispensable for the airway response. Therefore, MyD88 is involved, together with TIRAP, in TLR4 signaling resulting in acute pulmonary inflammatory response to local endotoxin, but the MyD88-dependent, IL-1 and IL-18 receptor signaling is dispensable for this response.

Molecular modeling has suggested that TIRAP, MyD88 and TLR4 receptor may form a heterotetrameric complex, where TLR4 homodimerises and which, together with accessory molecules such as LBP, MD2 and CD14, allowing LPS recognition and downstream signaling. The role of TIRAP in TLR4 signaling was believed to be structural, acting as a linker of MyD88 to TLR4, similar to TRAM that may act by bridging TRIF and TLR4. Both TIRAP and TRAM have been found to associate constitutively with TLR4^{22,42} and may serve as platform-forming components responsible for recruitment of the larger adaptors MyD88 and TRIF, respectively, that in turn, recruit downstream effector molecules such as IRAK-1 and IRAK-4 to MyD88 and TBK-1, TRAF6 and IKK- ϵ to TRIF via non-TIR domains. Nevertheless, TIRAP and MyD88 bind to different sites on TLR 2 and 4, a finding consistent with a cooperative role of the two adaptors in signaling. Moreover, a novel feature in TIRAP that distinguishes it from MyD88 was identified which implicates TIRAP in downstream signaling through the engagement of

distinct kinases. Indeed, putative TRAF6 interaction sites were identified in TIRAP but not in MyD88 suggesting a specific role of TIRAP in TLR2- and TLR4-mediated regulation of NF- κ B-dependent gene transcription via interaction of its TIR domain with TRAF6.⁴³ Interaction between TIRAP and TRAF6 is necessary for p65-mediated transactivation of NF- κ B, to control gene expression which is of critical importance to regulate the proinflammatory response. Transactivation by p65 is dependent of Bruton's tyrosine kinase (Btk), a critical tyrosine kinase in LPS signaling which has been shown to interact with, and phosphorylates TIRAP and TRAM.^{44–48} TIRAP was also recently implicated in negative regulation of TLR4 signaling. Membrane-bound form of ST2, a member of the TIR family that does not activate NF- κ B, was shown to negatively regulate TLR4-mediated NF- κ B activation by sequestering the adaptors TIRAP and MyD88, implicating TIRAP as a target for inhibition of TLR-4 signaling.⁴⁹ In the context of these new functions of TIRAP beyond the sole adaptor function, our study shows that TIRAP is an essential component for the LPS-induced MyD88-dependent inflammatory lung responses in mice and that MyD88 cannot compensate the lack of TIRAP.

Recent evidence suggests that TLR4 activation results in phosphorylation of TIRAP/Mal⁴⁴ and TRAM.⁴⁵ Further, LPS activation induced TIRAP/Mal localization to the plasma membrane by binding to PIP2 (phosphatidylinositol 4,5-bisphosphate) sites. This binding recruits MyD88 to TLR4, which is the first evidence of a cross-talk between TLR signaling and phospholipid metabolism.⁵⁰ Further, for the MyD88-independent pathway of TLR4 activation recent evidence suggests that TRAM containing a N-terminal myristoylation site is targeted to plasma membrane colocalized to TLR4.⁵¹ Upon activation membrane bound TRAM recruits TRIF to TLR4 and activates interferon regulatory factor 3 (IRF-3) and induced type 1 interferons and delayed NF- κ B activation.⁵² Therefore, at least for TLR4 signaling the role of the key adaptor proteins TIRAP/Mal and TRAM is getting clearer and completes the most recent reviews.^{16,17}

Although LPS induces a MyD88-independent response, which is TRIF-mediated, and result in type I interferon production *in vitro*,⁴¹ our data exclude a major role for TRIF-dependent, MyD88- and TIRAP-independent signaling mechanisms in the inflammatory lung response to endotoxin. Therefore, endotoxin-induced bronchoconstriction, injury of the alveolar epithelium and endothelium that result in neutrophil recruitment into the lung and the bronchoalveolar space, and vascular leak, are strictly dependent on the presence of both adaptor proteins, MyD88 and TIRAP but are independent of the type I interferon inducing, TRIF adaptor pathway.

In conclusion, our data demonstrate that both TIRAP and MyD88 adaptor molecules play a critical

and non-redundant role in bronchoconstriction, proinflammatory cytokine secretion, vascular leak and neutrophil recruitment in response to aerogenic LPS while TRIF-dependent signaling is dispensable for the endotoxin-induced airway response.

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Disclosures

The authors have no financial conflict of interest.

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