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Recruitment of distinct immune cell populations to the lung after intratracheal TLR4 signaling activation by two different stimulations

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The toll-like receptor 4 (TLR4)-mediated immune response is considered as one of the triggers of acute respiratory distress syndrome. The agonistic monoclonal antibody UT12 specific for the TLR4/MD2 complex induces immune activation in a manner distinct from lipopolysaccharide (LPS). In order to compare the effects of this differential TLR4 signaling activation, we examined immune cell recruitment to the lung following intratracheal inoculation with UT12 and LPS in mice. The increase in pulmonary neutrophils was much higher after LPS treatment compared with UT12 treatment, while $CD11b^{hi}CD11^{+}$cells increased to similar levels following both treatments. These changes were MyD88-dependent and TRIF-independent. These differential effects on immune cell recruitment to the lung suggest distinct underlying mechanisms in response to TLR4 stimulation. These findings further indicate that TLR signaling can lead to different outcomes depending on the ligand and activation pathway, which may relate to the complex pathogenesis of inflammatory lung diseases.

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Key words: lung, TLR4, neutrophils, dendritic cells

Introduction

Acute respiratory distress syndrome (ARDS) is a type of acute diffuse inflammatory lung injury leading to increased pulmonary vascular permeability, increased lung weight, and loss of aerated lung tissue that can require intensive care and in the worst case be lethal [1, 2]. The alveolar-capillary barrier consists of two separate barriers, the alveolar epithelium and the microvascular endothelium. Pulmonary edema is caused by either alveolar or vesicular injuries, although the mechanisms underlying the pathogenesis are not clearly understood. Direct injuries of the pulmonary epithelium such as that caused during pneumonia increase the permeability of lung microvessels, while indirect disorders such as sepsis or other severe inflammatory disorders within the microvessels result in injuries to vascular endocapillary cells and alveolar endothelial cells. Intra-tracheal administration of lipopolysaccharide (LPS), which induces the activation of innate immunity in intrabronchial cells, is often used as a model of ARDS [3]. LPS forms a complex with LPS-binding protein (LBP) and CD14 on the cell surface and stimulates the complex formation of toll-like receptor 4 (TLR4) with the lipid-binding protein MD2 [4, 5]. This receptor-mediated TLR4/MD2 clustering in lung cells, including pulmonary epithelial cells, dendritic cells (DCs), and macrophages, induces their production of cytokines and chemokines and recruits various immune cells to the pulmonary tissue, culminating in the development of ARDS-like diseases [6-9].

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UT12 is an agonistic monoclonal antibody (mAb) specific for the TLR4/MD2 complex, which stimulates the TLR4 signaling pathway through NF-κB activation and induces the production of pro-inflammatory cytokines [10, 11]. UT12 binds to the TLR4/MD2 complex and therefore by itself triggers the activation signals in a manner restricted only by this antibody-receptor complex. The TLR4-mediated immune response induced by UT12 is slightly different from that induced by LPS. LPS tolerance is transiently induced in animals administered with low doses of LPS, while UT12 can induce prolonged unresponsive state [10]. In the present study, we examined the mobilization of immune cells in the lung following TLR4 signaling via intratracheal stimulation by two distinct methods, namely administration of LPS or UT12.

Materials and Methods

Cell culture, ELISA, and antibodies

RAW264.7 cells (1×10^5) were cultured in a 96-well flat-bottom plate in the presence or absence of UT12 (0.001–10 μg/mL) and LPS (0.001–1 μg/mL, from *Pseudomonas aeruginosa* 10, Sigma-Aldrich, St. Louis, MO, USA) for 24 h. The IL-6 and IL-12 levels in the supernatant were determined by a sandwich ELISA as previously described [12].
For UT12 mAb collection, hybridoma cells were injected intraperitoneally into SCID mice and the IgG fraction was purified from the ascites using T-GEL MacroPAC (Scipac, Sittingbourne, UK) as previously described [10].

Animals

MyD88$^{-/-}$ and *TRIF*$^{-/-}$ mice were provided by Drs. S. Akira and T. Takeda (Osaka University, Osaka, Japan) [13, 14]. C57BL/6 (B6) mice were purchased from SLC (Shizuoka, Japan). The animals were maintained in the Laboratory Animal Center of Nagasaki University (LACNU). Mice were anesthetized by intraperitoneal administration of pentobarbital sodium salt (40 mg/kg), and the external tube of a 24G intravascular indwelling needle (BD Biosciences, San Diego, CA, USA) was inserted into the trachea as described previously with some modifications [15]. Saline solution (50 μL) containing UT12 (10 – 0.1 μg) or LPS (100 – 0.1 μg) was injected into the trachea. Mice were sacrificed 2 or 7 days after administration, and both lungs were collected. The animal experiments were approved by the Ethics Review Committee for Animal Experimentation of Nagasaki University, and were conducted in accordance with the guidelines for animal experimentation of LACNU.

Abbreviations: ARDS, acute respiratory distress syndrome; DCs, dendritic cells; LPS, lipopolysaccharide; mAb, monoclonal antibody

Cell preparation and flow cytometry

Lung tissue was immersed in RPMI1640 medium containing collagenase (10 mg/mL, SERVA, Heidelberg, Germany), finely minced, and incubated at 37 ℃ for 30 min as described previously [12]. After filtration and centrifugation, cells were treated with Gey's solution to lyse red blood cells, washed with RPMI1640, and resuspended in PBS containing 0.5 % BSA, 2 mM EDTA, and 0.09% sodium azide. Cells were stained with FITC-anti-CD11c, PE-anti-Ly6G (Gr-1), FITC-anti-F4/80, or FITC-anti-CD45R (B220) mAbs, or with biotin-anti-CD11b, biotin-anti-TCR β, and allophycocyanin-streptavidin (all from e-Biosciences, San Diego, CA, USA). After washing, 7-amino-actinomycin D (7AAD) was added to exclude dead cells, and samples were analyzed using FACSCanto II and FACSDiva™ Software (BD Biosciences).

Statistics

Data are expressed as mean ± standard deviation (SD). LPS-treated, UT12-treated and control groups were first compared by one-way analysis of variance (ANOVA) at a significance level of 0.05, and, if significant, Holm-Sidak's multiple comparisons test was used to draw comparisons between each pair in the group. Differences between two groups were analyzed by an unpaired *t*-test. A two-tailed *p*-value of less than 0.05 was considered significant. All statistical analyses were conducted using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA).

Results

Differential cellular responses to LPS and UT12

The RAW264 macrophage cell line was stimulated with different doses of LPS or UT12, and the production of cytokines *in vitro* was determined (Fig. 1). IL-6 and IL-12 levels in the supernatant increased in a dose-dependent manner in response to both LPS and UT12 treatment. Nonetheless, the maximum levels of IL-6 and IL-12 produced in response to LPS were approximately 14 and 6 times higher, respectively, than those produced in response to UT12. We next examined the effects of intra-tracheal LPS or UT12 adminis-

tration on lung infiltration of immune cells *in vivo* (Fig. 2). The number of infiltrating cells in the lung increased in a dose-dependent manner when the doses of LPS and UT12 were below 1 μg/mouse and plateaued at higher doses. The total number of infiltrating cells in response to LPS was generally higher compared with UT12, although the differences were not statistically significant. We focused on neutrophil ($Gr1^+F4/80^-$cells) infiltration because they represent the major cell type accumulating in the lung during ARDS [2]. The neutrophil proportion of all infiltrating cells was similar after administration of LPS or UT12 at doses below 1 μg/mouse. In contrast, the proportion of neutrophils increased in a dose-dependent manner at higher LPS doses, while it reached a maximum level at 1 μg/mouse of UT12 and did not increase at higher doses. In addition, the peak proportion of neutrophils in the lung-infiltrating cells of UT12-treated mice (approximately 47%) was lower than that of LPS-treated mice (approximately 75%).

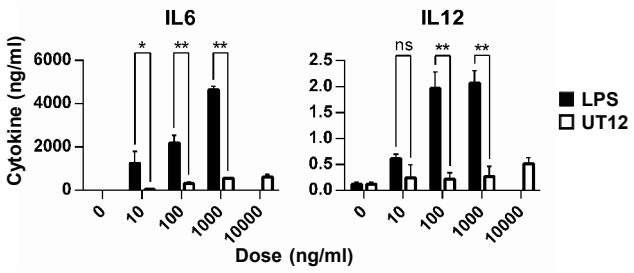

Figure 1 LPS- and UT12-induced cytokine production in the RAW264.7 macrophage cell line.

RAW264.7 cells were cultured for 24 h in the presence of different concentrations of UT12 (open bar) or LPS (closed bar). IL-6 and IL-12 levels in the supernatant were determined by ELISA. (Unpaired *t*-test: * $<$ 0.05, ** $<$ 0.01) Note that cytokine production by macrophage cell line in response to LPS was higher than that in response to UT12.

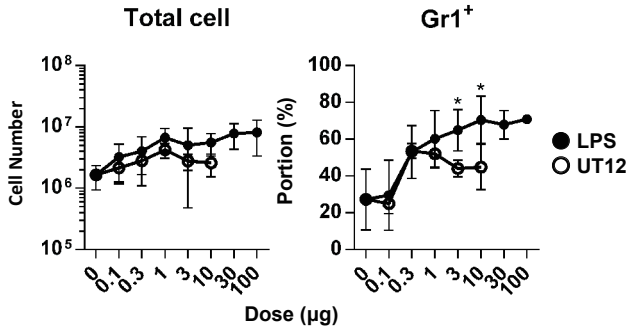

Figure 2 Increase in pulmonary infiltrating cells after intratracheal administration of LPS or UT12.

B6 mice received UT12 (closed circle) or LPS (open circle) by intratracheal administration. Mice were sacrificed 48 h later, and total numbers of cells extracted from the lung were determined (left panel). Lung cells were stained for granulocyte marker, Gr1, and the proportion of $Gr1^+$ cells (mostly neutrophils) in the total pulmonary cell population was determined by flow cytometry. Unpaired t test: * $<$ 0.05. Note that the number of neutrophils that are recruited into lung in response to LPS and UT12 was different.

Infiltrating cells in the lung after administration of LPS or UT12

Since the number of infiltrating cells in the lung reached maximum levels 2–3 days after LPS treatment (data not shown), we examined the infiltrating cells in more detail at 2 and 7 days after intra-tracheal administration of LPS or UT12. Cells were stained with their respective markers, and the proportions of $Gr1^+F4/80^-$ cells (neutrophils), $CD11b^{lo}$ $CD11c^+$cells, $CD11b^{hi}CD11c^+$cells, TCR β^+ cells (T cells), and $B220^+$ cells (B cells) were determined using flow cytometry (Fig. 3, 4). $CD11b^{lo}CD11c^+$cells are likely $CD11b^{lo}$ DCs, and $CD11b^{hi}CD11c^+$cells may include $CD11b^{hi}$ DCs, alveolar macrophages and monocyte-derived cells that are recruited to the tissue during inflammation [16, 17]. The total number of lung-infiltrating cells significantly increased in mice treated with LPS 2 days after administration (Fig. 4). In UT12-treated mice, there was a general trend towards increased cell numbers, but it was not statistically significant. The proportions of neutrophils and $CD11b^{hi}CD11c^+$cells (mostly monocyte-derived DCs and alveolar macrophages) relative to the total number of infiltrating cells increased significantly in both LPS- and UT12-treated mice. The increase in neutrophils was much higher in LPS-treated mice, while the number of $CD11b^{hi}CD11c^+$cells was similar between both treatments. The numbers of $CD11b^{lo}CD11c^+$cells (mostly conventional DCs) were not significantly different between the treatments, while their proportions in the total number of lung cells was reduced due to the increase in neutrophils and $CD11b^{hi}CD11c^+$cells. The number of T and B cells in the lung did not change 2 days after either treatment. Seven days after the stimulation, the proportions and absolute numbers of neutrophils, $CD11b^{lo}CD11c^+$cells, $CD11b^{hi}CD11c^+$cells, and lymphocytes (both T and B cells) in mice treated with either LPS or UT12 returned to levels similar to those before the treatment (Fig. 3, 4).

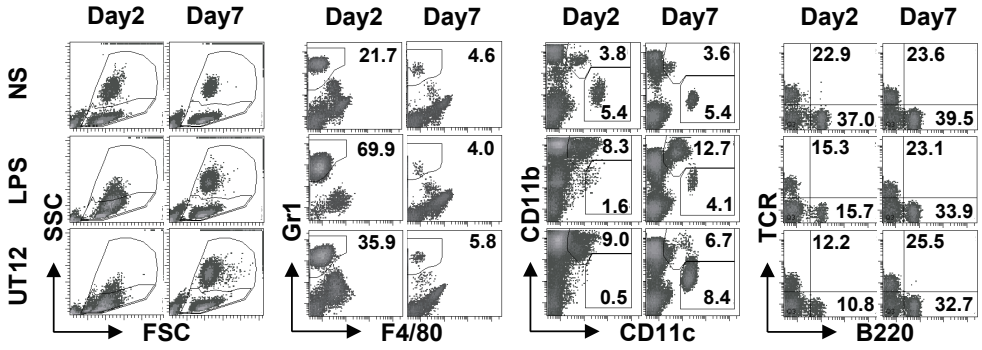

Figure 3 Flow cytometry analysis of lung-infiltrating cells after intratracheal administration of LPS or UT12.

B6 mice received saline (NS), LPS, or UT12 by intratracheal instillation. After 2 and 7 days, cells were extracted from lung tissue, stained for Gr1/F4/80, CD11b/CD11c, or TCR β /B220, and were analyzed by flow cytometry. Gr1/F4/80 and CD11b/CD11c are shown after large gating in FSC/SSC, and TCR β /B220 are shown after small lymphocyte gating in FSC/SSC. The numbers in the $Gr1^{+}/F4/80^{-}$ (neutrophils), $CD11b^{lo}/CD11c^{+}$ (mostly conventional DCs), and $CD11b^{hi}/CD11c^{+}$ (mostly monocyte-derived DCs and alveolar macrophages) flow cytometry profiles indicate the proportions (%) of gated cells in dot plots. The numbers in the TCR/B220 (T cells and B cells) profile indicate the proportions of cells in the upper left and lower right quadrants. Note that the proportions of neutrophils and $CD11b^{hi}/CD11c^{+}$ cells increased in LPS- and UT12-treated groups.

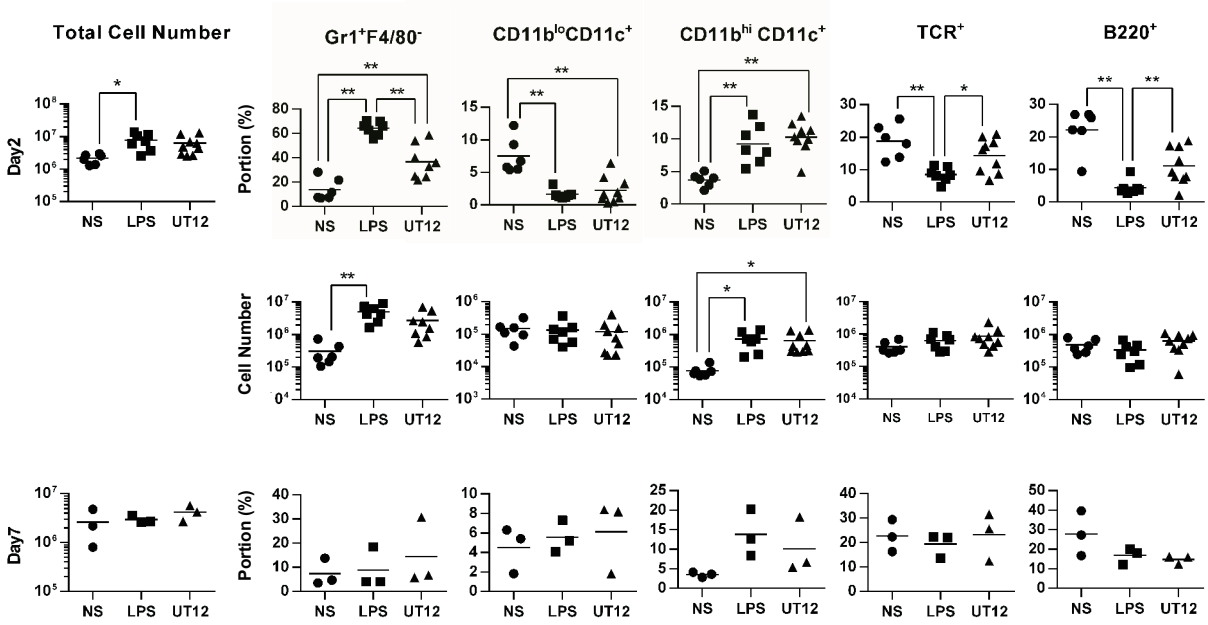

Figure 4 Pulmonary infiltrating cells after intratracheal administration of LPS or UT12.

B6 mice received saline (NS), LPS, or UT12 by intratracheal instillation. Lung-infiltrating cells were stained with mAbs 2 and 7 days after treatment and were analyzed by flow cytometry as shown in Figure 3. The proportions and total numbers of $Gr1^{+}F4/80^{-}$ (neutrophils), $CD11b^{lo}CD11c^{+}$ (mostly conventional DCs), $CD11b^{hi}CD11c^{+}$ (mostly monocyte-derived DCs and alveolar macrophages), $TCR^{+}B220^{-}$ (T cells), and $TCR^{-}B220^{+}$ (B cells) cells in each individual mouse were plotted. The horizontal bar in each graph indicates the mean value. ANOVA followed by Holm-Sidaks multiple comparisons test: * $<$ 0.05, ** $<$ 0.01. Note that the increase in neutrophil numbers in LPS-treated mice was larger than that in UT12-treated mice, while the increase in $CD11b^{hi}$ $CD11c^{+}$ cells was similar in both groups.

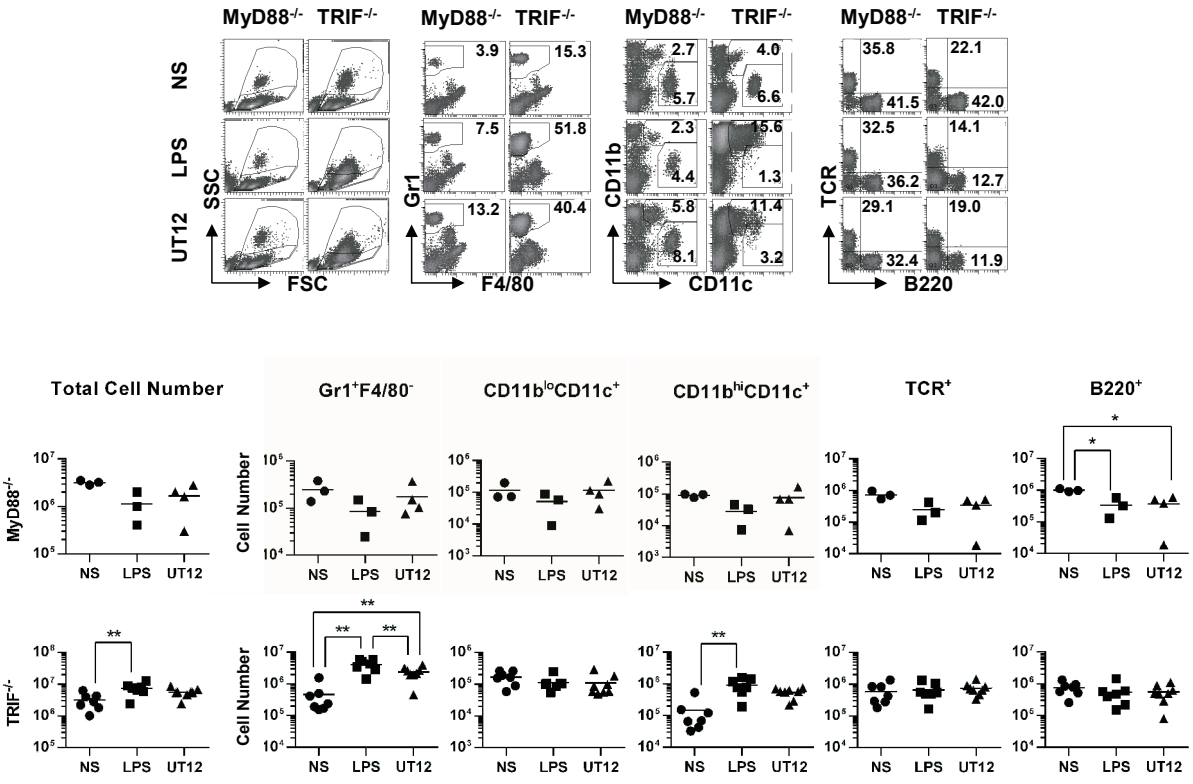

Figure 5 Pulmonary infiltrating cells did not increase in *MyD88*$^{-/-}$ mice after intratracheal administration of LPS or UT12.

MyD88$^{-/-}$ and *TRIF*$^{-/-}$ mice received saline (NS), LPS, or UT12 by intratracheal instillation. Two days after the treatment, lung-infiltrating cells were stained for Gr1/F4/80, CD11b/CD11c, or TCR β /B220, and were analyzed by flow cytometry as shown in Figure 3 (higher panel). $Gr1^{+}F4/80^{-}$, $CD11b^{lo}CD11c^{+}$, $CD11b^{hi}CD11c^{+}$, $TCR^{+}B220^{-}$, and $TCR^{-}B220^{+}$ cells represent mostly neutrophis, conventional DCs, monocyte derived DCs, T cells and B cells. The number of total lung cells and their subpopulations are plotted (lower panel). The horizontal bar in each graph indicates the mean value. ANOVA followed by Holm-Sidak's multiple comparisons test: * $<$ 0.05, ** $<$ 0.01. Note that the increase in neutrophils and $CD11b^{hi}CD11c^{+}$ cells was observed in *TRIF*$^{-/-}$ but not in *Myd88*$^{-/-}$ mice.

3.3 Infiltrating cells in the lung of *TRIF*$^{-/-}$ and *MyD88*$^{-/-}$ mice after administration of LPS or UT12

MyD88 and TRIF are adapter molecules involved in critical branching points of TLR4 signal transduction. The MyD88-mediated pathway induces cytokine production and expression of co-stimulatory molecules, while the TRIF-mediated pathway is critical for the induction of type I interferon [18]. It is controversial which signal transduction pathway is more important for the onset and exacerbation of ARDS [19-21]. To examine the roles of these adaptor molecules in immune cell infiltration of the lung, *MyD88*$^{-/-}$ and *TRIF*$^{-/-}$ mice were treated with LPS or UT12 via the intratracheal route. Two days after the administration, cells infiltrating the lung were analyzed using flow cytometry (Fig. 5). In *MyD88*$^{-/-}$ mice, the numbers of $Gr1^{+}F4/80^{-}$ cells (neutrophils), $CD11b^{lo}CD11c^{+}$cells (mostly conventional DCs), $CD11b^{hi}CD11c^{+}$cells (mostly monocyte-derived DCs and alveolar macrophages), and TCR^{+} cells (T cells) did not significantly increase in mice treated with either LPS or UT12, when compared with the control saline-treated group. In *TRIF*$^{-/-}$ mice, the total number of lung-infiltrating cells as well as those of $Gr1^{+}F4/80^{-}$ cells and $CD11b^{hi}CD11c^{+}$cells increased after administration of LPS or UT12, in a manner similar to those in B6 wild-type mice. These results suggest that the MyD88-mediated signaling pathway, but not the TRIF-mediated signaling pathway, plays a pivotal role in the recruitment of neutrophils and inflammatory cells to the lung tissue after intra-tracheal administration of both LPS and UT12.

Discussion

Instillation of LPS into the lung induces recruitment of immune cells to the pulmonary tissue. We investigated the regulation of this recruitment using two distinct stimulation

methods of TLR4 signaling, LPS and the anti-TLR4 mAb UT12 [10]. The macrophage cell line RAW 264.7 produced higher levels of cytokines in response to LPS than UT12 across a wide dose range, confirming the differences in cellular activation via TLR4 by these two distinct stimulations. Instillation of LPS induced recruitment of neutrophils in a dose-dependent manner, while the effect of UT12 reached a maximum level at 1μg and did not further increase at higher doses. LPS activates clustering of the TLR/MD2 complex in association with LBP and CD14 and thereby induces intracellular activation signals in lung cells such as pulmonary epithelial cells and macrophages, causing the recruitment of various immune cell populations to the pulmonary tissue [4, 5, 8, 9]. In contrast, UT12 triggers the activation signals in a manner restricted only by this antibody-receptor complex [11]. In addition, the IgG molecule is a relatively stable protein and has a long half-life in blood [22]. The differences in the TLR4 signaling activation by these molecules may determine the quality of pulmonary cell activation and culminate in distinct TLR4-mediated recruitment of neutrophils.

Two types of DCs exist in the lung, $CD103^{+}CD11b^{lo}$ DCs and $CD103^{-}CD11b^{hi}$ DCs, which differ in their tissue distribution, surface marker expression, and function [23-25]. $CD103^{+}CD11b^{lo}$ DCs reside in the lung mucosa and the vascular wall and are the main DCs to transport inhaled antigen to the draining lymph nodes for activation of specific T cells. $CD103^{-}CD11b^{hi}$ DCs are mainly present in perivascular regions of the lung and are the major producers of chemokines in the lung, both in homeostasis and under inflammatory conditions. In addition, monocyte-derived DCs are rapidly recruited to the lung upon inflammation [17, 26]. The number of $CD11b^{hi}CD11c^{+}$ cells increased after inoculation of UT12 and LPS, which is likely due to the recruitment of monocyte-derived DCs into the lung tissue. We did not observe significant differences in the number of $CD11b^{lo}CD11c^{+}$ cells, which are mostly resident conventional DCs, after administration of UT12 or LPS. Thus, the effect of TLR signaling on the recruitment of immune cells to the pulmonary tissue differs between cell types. Instillation of LPS induced a higher neutrophil recruitment than UT12, while both treatments induced similar numbers of monocyte-derived DCs ($CD11c^{hi}CD11c^{+}$ cells).

Chemokines and their receptors are known to play crucial roles in the recruitment of inflammatory cells to the lung [27]. The recruitment of neutrophils in response to TLR stimulation occurs in two phases [28]. The first wave peaks at day 1 and is dependent on CXCL1 and CXCL2, which both bind to chemokine receptor CXCR2 on neutrophils [29]. The second wave is a persistent influx of neutrophils, and may depend on CXCL12 binding to its receptor CXCR4 [30]. Monocyte-derived $CD11b^{hi}$ DCs express CCR6 and when recruited to lung tissue, they are induced to express CXCL12 and CCL20 after LPS exposure [26, 31]. Thus, one possibility to account for the differential effects of LPS and UT12 on the recruitment of neutrophils and monocyte-derived DCs is the difference in chemokine production of the lung. LPS may induce CXCL1, CXCL2, or CXCL12 at levels higher than UT12, recruiting more neutrophils, while both signals may induce similar levels of CCL20, therefore recruiting similar levels of monocyte-derived DCs. Alternatively, the levels of CCL20 induced by LPS and UT12 were initially different but reached a threshold level of CCR6 signaling, thus recruiting similar numbers of monocyte-derived DCs following both stimulations. Further studies on the chemokines and their receptors that are induced by LPS and UT12 may help our understanding of the differences and similarities of immune cell populations recruited to pulmonary tissue following administration of LPS or UT12.

TLR4 signaling branches in two pathways, which are mediated by different adapter molecules, MyD88 and TRIF [13, 14]. The MyD88 pathway is shared by all TLRs except TLR3, and induces cytokine production as well as activation of the antigen-presenting function. The TRIF-pathway activates IFN-β expression in response to TLR3 and TLR4 ligands [32]. The production of chemokines in response to TLR stimulation appears to be mediated by both MyD88- and TRIF-dependent pathways. The chemokines CXCL1 and CXCL2 that recruit neutrophils are both produced via the MyD88-dependent pathway, while only CXCL2 is induced through the TRIF-dependent pathway [29]. We used *MyD88*$^{-/-}$ and *TRIF*$^{-/-}$ mice to determine whether TLR4-mediated recruitment of immune cells to the lung tissue is mediated by the Myd88- or the TRIF-dependent pathway. On day 2 after LPS or UT12 administration, neutrophils and $CD11c^{hi}CD11c^{+}$ cells, mostly monocyte-derived DCs, were recruited to the lung in *TRIF*$^{-/-}$ mice similarly to the B6 wild-type mice. In contrast, the immune cell number did not increase in the lung of *MyD88*$^{-/-}$ mice after either treatment, suggesting that the recruitment of neutrophils and monocyte-derived DCs to the lung after both LPS and UT12 stimulation depends on the MyD88-mediated pathway.

In conclusion, we report that intratracheal instillation of LPS induced the recruitment of neutrophils at much higher levels compared with UT12 treatment, while the recruitment of monocyte-derived DCs did not significantly differ between both treatments. These features of immune cell recruitment to the lung by different TLR4 stimulations may help our understanding of the cellular and molecular mecha-

nisms underlying accumulation of immune cells in the lung that ultimately lead to the pathogenesis of immune response-mediated lung diseases such as ARDS.

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