Tetraspanin CD9 Negatively Regulates Lipopolysaccharide-Induced Macrophage Activation and Lung Inflammation

Mayumi Suzuki, Isao Tachibana, Yoshito Takeda, Ping He, Seigo Minami, Takeo Iwasaki, Hiroshi Kida, Sho Goya, Takashi Kijima, Mitsuhiro Yoshida, Toru Kumagai, Tadashi Osaki and Ichiro Kawase

*J Immunol* 2009; 182:6485-6493; doi: 10.4049/jimmunol.0802797
http://www.jimmunol.org/content/182/10/6485

**References**
This article cites 30 articles, 12 of which you can access for free at:
http://www.jimmunol.org/content/182/10/6485.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Tetraspanin CD9 Negatively Regulates Lipopolysaccharide-Induced Macrophage Activation and Lung Inflammation

Mayumi Suzuki,* Isao Tachibana,2* Yoshito Takeda,* Ping He,†† Seigo Minami,* Takeo Iwasaki,* Hiroshi Kida,* Sho Goya,* Takashi Kijima,* Mitsuhiro Yoshida,* Toru Kumagai,* Tedashi Osaki,* andIchiro Kawase*

Tetraspanins facilitate the formation of multiple molecular complexes at specialized membrane microdomains and regulate cell activation and motility. In the present study, the role of tetraspanin CD9 in LPS-induced macrophage activation and lung inflammation was investigated in vitro and in vivo. When CD9 function was ablated with mAb treatment, small interfering RNA transfection, or gene knockout in RAW264.7 cells or bone marrow-derived macrophages, these macrophages produced larger amounts of TNF-α, matrix metalloproteinase-2, and -9 upon stimulation with LPS in vitro, when compared with control cells. Sucrose gradient analysis revealed that CD9 partly colocalized with the LPS-induced signaling mediator, CD14, at low-density light membrane fractions. In CD9 knockout macrophages, CD14 expression, CD14 and TLR4 localization into the lipid raft, and their complex formation were increased whereas IκBα expression was decreased when compared with wild-type cells, suggesting that CD9 prevents the formation of LPS receptor complex. Finally, deletion of CD9 in mice enhanced macrophage infiltration and TNF-α production in the lung after intranasal administration of LPS in vivo, when compared with wild-type mice. These results suggest that macrophage CD9 negatively regulates LPS response at lipid-enriched membrane microdomains. The Journal of Immunology, 2009, 182: 6485–6493.

Lung inflammation, which is characterized by the infiltration of inflammatory cells including alveolar macrophages and their production of proinflammatory mediators such as cytokines, oxidants, and proteases, underlies the pathophysiology of many respiratory diseases such as acute lung injury, interstitial pneumonia, granulomatous lung disease, and pulmonary emphysema. LPS, a major component of the Gram-negative bacteria cell wall, is a potent inducer of lung inflammation (1). It stimulates macrophages to produce cytokines and increase the expression of cell adhesion molecules. LPS binding up-regulates the expression of its receptor, CD14, and recruits CD14 and TLR4 into cholesterol and sphingolipid-enriched membrane microdomains designated the lipid raft, where clustering of the CD14/TLR4 receptor complex activates MAPK and NF-κB pathways and leads to rearrangement of actin cytoskeleton (2–5). Although signaling molecules essential for LPS-induced macrophage activation have been well characterized (1), factors modifying the level of LPS-induced lung inflammation still remain to be studied. Search for such factors might help to elucidate the pathogenesis of respiratory diseases involving lung inflammation.

Tetraspanin is a protein family comprising at least 33 members such as CD9, CD63, CD81, CD82, and CD151 in mammals. Its structure spanning the membrane four times endows this protein with a propensity to associate with each other, other tetraspanin members, and functional proteins such as integrins, growth factors, human leukocyte Ags, and intracellular signaling molecules (6). At specialized membrane microdomains, designated tetraspanin-enriched microdomain (TEM)3, tetraspanins facilitate the formation of these multimolecular complexes and thereby regulate cell activation, fusion, motility, and signaling (7). Although proteins at both rafts and TEMs can distribute into low-density light membrane fractions in sucrose gradient analysis, these microdomains are thought to be distinct in view of several aspects (8). However, recent studies have suggested that there may be an overlapping or an interaction between raft and TEM proteins. Upon stimulation of macrophages with RANKL, CD9 was localized into the lipid raft (9). Also, it was proposed that TEMs and lipid rafts may associate under certain conditions, resulting in the close proximity of a distinct set of signaling molecules in platelets (10).

We recently reported that, in mice doubly deficient in tetraspanins CD9 and CD81, macrophages infiltrated into the lung and pulmonary emphysema spontaneously developed (11, 12). This observation was not obvious in mice lacking CD9 or CD81 alone (12) and thus suggests that these tetraspanins coordinately prevent lung inflammation. However, its detailed mechanisms have yet to be clarified, and whether the loss of CD9 or CD81 alone can cause the lung inflammation under certain conditions still remains unknown. In the present study, we show that, after stimulation with LPS, the loss of CD9 function enhances macrophage activation in vivo.

Received for publication August 29, 2008. Accepted for publication March 13, 2009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked
with 18 U.S.C. Section 1734 solely to indicate this fact.

3 Abbreviations used in this paper: TEM, tetraspanin-enriched microdomain; SIRPα, signal regulatory protein α; 2OHpCD, 2-hydroxypropyl-β-cyclodextrin; KO, knockout; BMDM, bone marrow-derived macrophage; WT, wild type; BALF, bronchoalveolar lavage fluid; siRNA, small interfering RNA; MMP, matrix metalloproteinase.

Copyright © 2009 by The American Association of Immunologists, Inc. 0022-1767/99/$2.00

The Journal of Immunology

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0802797
vitro and exacerbates lung inflammation in vivo. We also propose mechanisms by which CD9 negatively regulates LPS-induced signaling. CD9 prevents CD14-dependent receptor assembly at lipid-enriched membrane microdomains.

Materials and Methods

Abs and reagents

Rat anti-mouse CD9 mAb (KMC8) and hamster anti-mouse CD81 mAb (Ea2) were purchased from BD Biosciences and U.K.-Serotec, respectively. Rat anti-CD14 mAb (rmC5–3) and goat biotinylated anti-CD14 polyclonal Ab (BAF9892) were purchased from BD Biosciences and R&D Systems, respectively. Rat anti-TLR4/MD2 complex mAb (38B4) and rabbit anti-CD9 mAb (IMG577) were obtained from BioLegend and IMGENEX, respectively. Mouse anti-flotillin-1 mAb (clone 18) and goat anti-CD45 polyclonal Ab were purchased from BD Biosciences and R&D Systems, respectively. Rabbit anti-IκBα polyconal Ab (9242) and anti-phosphorylated p38 polyclonal Ab (9211) were both from Cell Signaling Technology. Rabbit anti-p38 polyclonal Ab (sc-7149) and rabbit anti-SHP-1 polyclonal Ab (sc-287) were both obtained from Santa Cruz Biotechnology. Mouse anti-phosphotyrosine mAb (PY-20) and rabbit anti-signal regulatory protein α (SIRPα) polyclonal Ab (06–729) were purchased from BD Biosciences and Upstate Biotechnology, respectively. Phenol-extracted LPS from Escherichia coli O55:B5 was purchased from Sigma-Aldrich. HRP-conjugated cholera toxin B subunit was purchased from List Biological Laboratories. 2-hydroxypropyl-β-cyclodextrin (2OHpβCD) and an inhibitor of IκB kinase, BMS434541, were obtained from Calbio Tesque and Sigma-Aldrich, respectively. Cholesterol and methyl-β-cyclodextrin were both purchased from Sigma-Aldrich.

Mice

CD9 knockout (KO) mice were provided by Dr. E. Mekada (Research Institute for Microbial Diseases, Osaka University, Osaka, Japan) (13). These mice were backcrossed >6 generations into the C57BL/6J background. All experiments were performed in accordance with the Osaka University guidelines on animal care. Nine- to 12-wk-old CD9 KO mice and wild-type (WT) littermates matched for age and sex were used in all experiments.

Cell culture and stimulation with LPS

A mouse macrophage line, RAW264.7, was cultured in DMEM containing 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Mouse bone marrow-derived macrophages (BMDMs) were prepared as previously described (14). In brief, cells were isolated by flushing the bone marrow of tibias and femurs and cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Mouse alveolar macrophages were obtained by bronchoalveolar lavage. Lungs of mice were used for lysis and culture for a gradient of 0.1% Coomassie brilliant blue R250. The intensity of the bands was quantified with the FluorChem. Gelatinolytic bands were visualized by staining with 0.1% Coomassie brilliant blue R250. The intensity, lipid-enriched, light membrane fractions (fractions 4 and 5) or dense membrane fractions (fractions 6 and 7) were collected with protein G-Sepharose (Amersham Biosciences and Upstate Biotechnology, respectively). Materials and methods

siRNA duplexes targeting mouse CD9 (SMF27B-0251) were synthesized by and purchased from B-Bridge international. RAW264.7 cells (2.5 × 10⁶/ml) were transfected with either 40 μM mixture of the siRNAs or control random RNAs (S30C-0126; B-Bridge International) using LipoFECTAMINE 2000 reagent (Invitrogen). The cells were cultured for 3 days and gene-silencing effect was assessed by immunoblotting with anti-CD9 mAb. In some experiments, the cells were stimulated with 0.1 μg/ml LPS after 2 days of transfection.

Gelatin zymography

Samples containing equal amounts of protein from culture supernatants or bronchoalveolar lavage fluid (BALF) were electrophoresed on a 10% polyacrylamide gel containing 0.1% gelatin (Invitrogen). The gels were washed with 2.5% Triton X-100 and incubated at 37°C overnight in Novex zymogram developing buffer (Invitrogen). Gelatinolytic bands were visualized by staining with 0.1% Coomassie brilliant blue R250. The intensity of the bands was quantified with the FluorChem.

RT-PCR

Total RNA was extracted with Isogen (Nippon Gene), and 1 μg RNA was subjected to RT-PCR amplification using the following oligonucleotide primers: 5′-CCACCGCTGTGGTGTCGCCACAGTTG-3′ and 5′-GAAGTGTGCCGCTGCCACAGGAA-3′ for matrix metalloproteinase (MMP)-2 (16); 5′-TGATGGCTCGCCAGACATCCTGTC-3′ and 5′-CTTATCCACCGGAATGACGCTCT-3′ for MMP-9 (16). The thermal cycling parameters were 30 cycles of 30 s at 94°C, 30 s at 60°C, and 60 s at 72°C. We confirmed that these variables yielded the amplification of template DNAs within a linear range.

Sucrose gradients

BMDMs were lysed in 500 μl of MES buffer (150 mM NaCl and 20 mM MES (pH 6.5)) supplemented with 1% Triton X-100, 2 ml PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin for 1 h on ice. Lysates were then sheared by successive passage through hypodermic needles (5 × 18G1/2, 10 × 26G1/2). The lysate was mixed with an equal volume of 90% sucrose in MES buffer, placed at the bottom of a centrifuge tube, overlaid with 4.5 ml of 30% sucrose and 3.5 ml of 5% sucrose in MES buffer. After centrifugation at 100,000 × g for 16.5 h at 4°C in a Beckman SW40Ti rotor, fractions of 1 ml each were collected from the top of the gradient. Each fraction was added with 60 μM n-octyl-glucoside and analyzed by SDS-PAGE using 5–20% gradient gels (Wako Pure Chemical). Protein distribution in the fractions was visualized by immunoblotting with anti-CD14 (rmC5–3), anti-TLR4 (IMG577), anti-CD9, anti-CD81, anti-CD45, and anti-flotillin-1 Abs. GM1 ganglioside was detected with HRP-conjugated cholera toxin by dot blot using equal amounts of each fraction. The density of the gels was quantified with the FluorChem. In some experiments, low-density, lipid-enriched, light membrane fractions (fractions 4 and 5) or dense fractions (fractions 9 and 10) were pooled and subjected to immunoprecipitation using anti-CD9 mAb. Immune complexes were electrophoresed on SDS-PAGE and probed with biotinylated anti-CD9 mAb, followed by streptavidin-conjugated peroxidase.

LPS challenge in vivo

In brief, 0.5 mg/kg LPS in 40 μl sterile PBS was intranasally administered to anesthetized mice. After 2 h–4 days, bronchoalveolar lavage was performed twice with 0.5 ml PBS. The recovered BALF was centrifuged and the supernatant was analyzed for cytokines and MMP activities. Cell pellets were resuspended in PBS, and total cell count and its subset analysis were performed using a hemocytometer and Diff-Quick stain (Sysmex), respectively. In another experiment, the cell pellets were resuspended in RPMI 1640 containing 2% FBS and cultured for 5 days. The cells were photographed, and morphologically activated macrophages were quantified by determining the percentage of spread cells with a process longer than 1-cell diameter (17).

Cytokine analysis

Concentrations of TNF-α in culture supernatants or BALF were measured by ELISA using Quantikine (R&D Systems). Multiple cytokine analysis of BALF was performed using mouse cytokine antibody array (MA6412; Panomics) according to the manufacturer’s instructions. The signals were analyzed using the FluorChem.
Statistical analysis

In vitro assays were performed in quadruplicate cultures. Animal experiments were done using at least four mice for each group. All numerical results are expressed as mean ± SEM. Statistical differences were determined by Student’s t test. p values <0.05 were considered statistically significant.

Results

Expression of macrophage CD9 and CD81 in the absence or presence of LPS

Signaling molecules in LPS-induced macrophage activation have been well characterized (1), but involvement of tetraspanin CD9 has not been studied. We first investigated macrophage expression of CD9 and its closely related tetraspanin, CD81, in the absence or presence of LPS. When a mouse macrophage line, RAW264.7, was treated with 0.1 μg/ml of LPS, cellular activation was noticeable with their morphological change (Fig. 1A). The cells were spread and extended long projections with time, whereas such prominent changes were not observed in the absence of LPS. Expressions of CD9 and CD81 were confirmed by immunoblotting, and it appeared that protein levels of CD9 and CD81 were transiently down-regulated in the presence of LPS, whereas their levels were relatively unchanged in the absence of LPS (Fig. 1B). We next isolated BMDMs from mice and examined the expression of CD9 and CD81. These tetraspanins were present in BMDMs, and LPS again down-regulated their levels moderately (Fig. 1C). We further isolated mouse alveolar macrophages by bronchoalveolar lavage and tested the effect of LPS in culture. Because the number of isolated cells was not enough for immunoblotting, the expression of CD9 and CD81 was examined by flow cytometry; the levels of CD9 and CD81 in mean fluorescence intensity were 136 and 45 before, and reduced to 112 and 36 at 48 h after LPS stimulation, respectively.

The loss of CD9 function exacerbates LPS-induced TNF-α and MMP production in vitro

LPS-induced macrophage activation leads to the production of proinflammatory mediators including cytokines and proteinases through the activation of NF-κB (1, 18). To elucidate a role of CD9 in LPS signaling, we negated CD9 function of RAW264.7 with mAb or siRNA transfection and examined the production of TNF-α and MMPs in vitro. As shown in Fig. 2A, treatment of RAW264.7 cells with a function-blocking anti-CD9 mAb, KMC8 (11), augmented LPS-induced secretion of TNF-α when compared with isotype-matched IgG. KMC8 also increased the basal level of TNF-α in the absence of LPS. siRNA transfection against CD9, which successfully knocked down CD9 protein in RAW264.7, likewise enhanced LPS-induced TNF-α secretion as well as its basal level (Fig. 2B). In addition, the CD9 knockdown increased LPS-induced MMP-9 activity in culture supernatants as shown in gelatin zymography and its densitometry analysis (Fig. 2C). MMP-2 activity was not detected in this cell line (19).

To extend the role of CD9 to primary macrophages, similar experiments were performed using BMDMs from WT and CD9 KO mice. These cells were stimulated with LPS and the production of TNF-α and MMPs was examined. Like the mAb- or siRNA-treated RAW264.7 cells, CD9 KO BMDMs secreted more TNF-α than WT counterparts when activated with LPS (Fig. 3A). By contrast with RAW264.7, these cells did not increase the basal level of TNF-α, possibly reflecting the difference between a cell line and primary cells. Meanwhile, the basal and LPS-induced expressions of MMP-2 and MMP-9 were both up-regulated in CD9 KO BMDMs when compared with WT cells in RT-PCR (Fig. 3B). Augmentation of the LPS-induced gelatinolytic activity of MMP-2 and MMP-9 was confirmed by zymography (Fig. 3C). We additionally tested alveolar macrophages isolated from BALF and again found that CD9 KO macrophages produce a higher level of TNF-α when activated with LPS in vitro (Fig. 3D).

CD14 expression and its association with TLR4 are enhanced in CD9 KO macrophages

CD14 is a major component of the LPS receptor complex, and it was previously reported that LPS binding to macrophages up-regulates the expression of CD14 (5). To explore mechanisms underlying the loss of CD9 function effect on LPS signaling, CD14
expression and its association with TLR4 were examined using BMDMs isolated from WT and CD9 KO mice. As shown in Fig. 4A, protein level of CD14 was slightly increased in naive CD9 KO mice. As shown in Fig. 4A, protein level of CD14 was slightly increased in naive CD9 KO mice.
BMDMs, and LPS-induced CD14 up-regulation was enhanced compared with WT cells. Although the level of TLR4 remained unchanged (Fig. 4A), it coprecipitated a larger amount of CD14 protein in CD9 KO BMDMs after LPS stimulation (Fig. 4B, upper panel), most likely reflecting the increased expression of CD14 (Fig. 4B, middle panel) and its concentration to lipid rafts (see below).

**Distribution of CD14 and TLR4 to the lipid raft is increased in CD9 KO macrophages**

CD14 resides in lipid rafts in naive macrophages and, upon stimulation with LPS, more CD14 protein concentrates into the lipid rafts to form activation clusters with other signaling molecules including TLR4 (3, 4). Tetraspanins form their own multimolecular networks, TEM, which can interact with lipids, and thus tetraspanin complexes also appear within low-density light membrane fractions in sucrose gradients, especially when mild nonionic detergents are used (8). To study the localization into lipid and nonlipid microdomains before and after LPS stimulation, CD14, TLR4, CD9, and CD81 were immunoblotted using sucrose gradient fractions of BMDMs. In naive WT cells, CD14 resided both in light membrane fractions (fractions 4 and 5) and in dense fractions (fractions 9 and 10) (Fig. 4C, CD14, left upper panel). Proteins, although not all, in the former fractions were generally regarded as components of lipid rafts, as evidenced by the distribution of flotillin-1 and GM1 ganglioside into these fractions (Fig. 4C, flotillin-1 and GM1, left panels) (20, 21). Meanwhile, most TLR4 protein was distributed to dense fractions (Fig. 4C, TLR4, upper panel). Upon LPS stimulation, portions of CD14 (Fig. 4C, CD14, left lower panel) and TLR4 (Fig. 4C, TLR4, left lower panel) were recruited from dense fractions to light membrane fractions as previously described (21, 4). Most CD9 and CD81 proteins were solubilized and localized at dense fractions in naive BMDMs, because a stringent nonionic detergent Triton X-100 was used (Fig. 4C, CD9 and CD81, left upper panel) (22). However, upon LPS stimulation, these tetraspanins were partly redistributed to light fractions like TLR4 (Fig. 4C, CD9 and CD81, left lower panels).

We next tested sucrose gradient fractions from CD9 KO BMDMs. Of note, when CD9 was completely absent, a larger portion of CD14 protein was already localized into light membrane fractions (Fig. 4C, CD14, right upper panel) compared with WT (Fig. 4C, CD14, left upper panel) even in naive BMDMs, and LPS stimulation slightly up-regulated CD14 of these fractions (Fig. 4C, CD14, right lower panel). The loss of CD9 also shifted the distribution of TLR4 and CD81 to light fractions (Fig. 4C, TLR4 and CD81, right upper panels) compared with WT BMDMs (Fig. 4C, TLR4 and CD81, left upper panels) before LPS stimulation. As a result, probably, more TLR4 and CD81 proteins localized into light membrane fractions (Fig. 4C, TLR4 and CD81, left lower panels) after LPS stimulation. Densitometry supplements the immunoblots quantitatively indicating the shift of these proteins to light membrane fractions (Fig. 4C, LM % density units). By contrast, the distribution of GM1 ganglioside and a raft-marker protein, flotillin-1, was not affected by the absence of CD9 (Fig. 4C, flotillin-1 and GM1, right panels) when compared with WT (Fig. 4C, GM1 and flotillin-1, left panels). Also, the loss of CD9 did not affect the distribution of a nonraft protein, CD45 (23) (Fig. 4C, CD45, right panels), compared with WT (Fig. 4C, CD45, left panels). The codistribution of CD9 and CD14 into light membrane fractions, especially after LPS stimulation, raises a possible interaction between these two molecules in WT macrophages. Thus, we performed coprecipitation experiments using pooled light membrane fractions and dense fractions. Of note, more CD14 protein coprecipitated with CD9 in the light fractions than in the dense fractions before and after LPS stimulation (Fig. 4D), suggesting physical
proximity between CD9 and CD14 in Triton X-100 lysate. These results suggest that CD9 may interfere with CD14 and the CD9 deficiency facilitates CD14-mediated receptor assembly at the lipid-enriched microdomain.

**IkB degradation is accelerated in CD9 KO macrophages**

Signals from LPS-induced CD14/TLR4 receptor complex lead to the degradation of IkB and result in release and translocation of NF-κB to the nucleus, where it activates proinflammatory genes including TNF-α and IL-6 (1). To examine whether CD9 prevents this LPS-induced NF-κB activation, protein level of IkBα was investigated by immunoblotting. As shown in Fig. 5A, IkBα was decreased before and degraded earlier after LPS stimulation in CD9 KO BMDMs than in WT cells, suggesting that CD9 deficiency accelerates NF-κB-mediated inflammatory response. Because LPS binding to macrophages also causes translocation of MAPK including ERK2 and p38 to the lipid raft and results in their activation (1, 21), we examined LPS-induced p38 phosphorylation in parallel. However, no difference was observed between WT and CD9 KO BMDMs (Fig. 5A).

SIRPα, a member of SIRP family, is abundantly expressed in macrophages and regulates LPS-induced macrophage activation and lung inflammation through association with a phosphotyrosine phosphatase, SHP-1 (24, 25). We additionally examined the activation of these signaling molecules in WT and CD9 KO BMDMs. No obvious difference was detected in phosphorylation of SHP-1 and its association with SIRPα. However, the total level of SIRPα protein appeared to be slightly lower in CD9 KO BMDMs throughout the LPS stimulation by unknown mechanisms (Fig. 5B).

**Disruption of lipid-rich microdomains negates the enhanced TNF-α production in CD9 KO macrophages**

β-cyclodextrins are a class of heptasaccharides commonly used to selectively remove cholesterol from cellular membranes, and par-

![FIGURE 5.](http://www.jimmunol.org/Download/all/6490_5.png)  
**FIGURE 5.** Accelerated IkB degradation and analysis of other signaling molecules in LPS-activated CD9 KO BMDMs. **A,** BMDMs from WT and CD9 KO mice were stimulated with 1 μg/ml LPS. After the indicated minutes, cell lysates were separated by SDS-PAGE, transferred to a membrane, and immunoblotted with anti-IkBα, anti-phosphorylated p38 (p-p38), and anti-p38 polyclonal Abs. Anti-actin blots show comparable amounts of protein loaded in each lane. **B,** SHP-1 was immunoprecipitated using anti-SHP-1 polyclonal Ab from cell lysates of WT and CD9 KO BMDMs stimulated with LPS for the indicated hours. After electrophoresis on SDS-PAGE and transfer to a membrane, tyrosine-phosphorylated SHP-1 was probed with anti-phosphotyrosine mAb (PY20), and the associated SIRPα was blotted with anti-SIRPα polyclonal Ab. SHP-1 and SIRPα in whole cell lysates (WCL) were immunoblotted in parallel. Anti-actin blots show comparable amounts of protein loaded in each lane. Data shown are from one representative of three similar experiments.

**FIGURE 6.** Cholesterol depletion inhibits LPS-induced, NF-κB-dependent production of TNF-α. A, BMDMs from WT and CD9 KO mice were untreated (None) or pretreated with 10 mM 2OHp/CD or 1 μM BMS345541 and cultured in the absence (−) or presence (+) of 1 μg/ml LPS. After 24 h, the concentration of TNF-α in culture supernatants was measured in ELISA. B, BMDMs were untreated (None), pretreated with 10 mM 2OHp/CD (2OHp/CD), or reloaded with cholesterol after the 2OHp/CD treatment (2OHp/CD + cholesterol), and then stimulated with LPS. After 24 h, the concentration of TNF-α in culture supernatants was measured in ELISA. The assays were done using BMDMs collected from four mice for each group. Each bar represents the mean ± SEM. *p < 0.05 vs WT.
microdomains provide an essential platform for CD9 to modulate LPS-induced macrophage activation.

The loss of CD9 exacerbates LPS-induced lung inflammation and production of cytokines and MMPs in vivo

Last, we investigated whether CD9 prevents LPS-induced lung inflammation in vivo. CD9 KO mice and WT littermates were challenged with intranasal administration of PBS or 0.5 mg/kg LPS. After 4 days, the lungs were lavaged, and total cell count and its subset analysis in BALF were performed using a hemocytometer and Diff-Quick stain, respectively. The assays were done using BALF from nine mice for each group (n = 9). After 2 days of the LPS administration to WT and CD9 KO mice, macrophages in the BALF were further cultured for 5 days and photographed (upper panels). The percentage of spread macrophages with a process longer than 1-cell diameter was determined (lower panel). The assays were done using BALF from four mice for each group (n = 4). The photos are from one representative of each group. Bar, 50 μm. C. After 2 h of the PBS or LPS administration to WT and CD9 KO mice, the concentration of TNF-α in the BALF was measured in ELISA. The assays were done using BALF from six mice for each group (n = 6). D. After 2 days of the LPS administration to WT and CD9 KO mice, MMP-2 and MMP-9 gelatinolytic bands were visualized by Coomassie brilliant blue R250 staining (upper panel), and the intensity of lytic bands of MMP-2 and MMP-9 was quantified by densitometry (lower panel). E. After 2 h of the PBS or LPS administration to WT and CD9 KO mice, multiple cytokines in the BALF were analyzed using mouse cytokine Ag array (upper panels) and its densitometry (lower panels). The position of cytokines in each array was shown in the upper right panel. PC, positive control; NC, negative control. Percentage of density units in lower panels were determined when the density of PC and NC was set at 100 and 0 units, respectively. The assays were done using BALF from four mice for each group (n = 4) and in duplicate for each mouse. The upper left panels are from one representative of each group. Each bar represents the mean ± SEM. *, p < 0.05 vs WT; **, p < 0.01 vs WT.
mice compared with WT mice (Fig. 7B). The concentration of TNF-α in BALF revealed 2-fold increase in CD9 KO mice at 2 h after the LPS stimulation (Fig. 7C). Gelatin zymography using the BALF showed enhanced MMP-2 and MMP-9 activities in CD9 KO mice (Fig. 7D). We further evaluated expression profiles of multiple cytokines in the BALF using Cytokine Antibody Array. Consistent with the results of ELISA (Fig. 7C), a 2-fold increase in TNF-α was observed after the LPS administration in CD9 KO mice (Fig. 7E). Other macrophage cytokines including MIP-1α, G-CSF, IL-6, and IL-12 also appeared to be increased, although not statistically significant (p < 0.1 vs WT in all these cytokines). These results indicate that LPS-induced lung inflammation was exacerbated in CD9 KO mice compared with WT mice.

Discussion

In the present study, we firstly examined the expressions of tetraspanins CD9 and CD81 in cultured macrophages. The expression pattern was relatively different between the absence and presence of LPS (Fig. 1); when treated with LPS, these tetraspanins were transiently and moderately down-regulated in a cell line, RAW264.7, and primary macrophages. This down-regulation was concurrent with morphological activation. Based on the dogma that the tetraspanins form complexes with integrins and regulate cellular events involving cytoskeletal reorganization such as cell fusion and motility (7), we speculated that CD9 and CD81 may play a role in LPS-induced macrophage activation. To further clarify the role of the tetraspanins, we negated function of CD9 by mAb and siRNA treatment of RAW264.7 (Fig. 2) or by using KO macrophages (Fig. 3) and assessed the LPS response in vitro. In either case, LPS-induced production of proinflammatory mediators, TNF-α and MMPs, was augmented. Furthermore, in vivo deletion of CD9 in mice exacerbated LPS-induced lung inflammation, which was characterized by the infiltration of activated macrophages and overproduction of TNF-α and MMPs (Fig. 7). These results indicate that macrophage CD9 plays a negative role in the LPS response.

It has been recognized that membrane microdomains are important in LPS signaling (3). LPS stimulation of macrophages induces translocation of CD14 to the lipid raft and facilitates the formation of multimeric receptor complexes containing CD14, TLR4, and CD11b/CD18 (21). Meanwhile, tetraspanins including CD9 and CD81 also facilitate the formation of their own multimolecular protein network, designated TEM, at lipid-enriched microdomains. Although protein components at the raft and TEM can be localized at low-density light membrane fractions in sucrose gradients, it is thought that these two microdomains are distinct in view of several aspects. For example, lipid rafts are disrupted at 37°C, whereas TEMs are maintained. Lipid rafts are typically insoluble in non-ionic detergents, whereas TEMs are mostly soluble in nonionic detergents (8). However, recent data suggest that there may be an overlapping or an interaction between the raft and TEM proteins under certain conditions (9, 28, 10). Such interactions may result in the close proximity of these distinct sets of membranous molecules and facilitate signal transduction (10). Indeed, regulations of the lipid raft and TEM were both shown to be dynamic upon cellular activation in previous studies (3, 11). In the present study, most CD9 and CD81 proteins were soluble in Triton X-100 and distributed in dense sucrose gradient fractions, and a small portion of these protein resided in light fractions in naive BMDMs. Importantly, upon LPS stimulation, more CD9 and CD81 protein concentrated into the light fractions, where raft proteins also assemble. The coprecipitation experiments revealed a physical proximity between CD9 and CD14 in the light fractions but not in the dense fractions (Fig. 4). Thus, there might be a specialized lipid microdomain, where the raft and TEM interact, especially upon LPS stimulation.

The experiments using CD9 KO macrophages indicated that the loss of CD9 enhances LPS-induced signaling in at least two different ways (Fig. 4). First, the protein level of CD14 was increased in naive CD9 KO BMDMs, and its up-regulation with LPS was enhanced when compared with WT cells. LPS-induced increase of CD14 expression occurs at the mRNA level in RAW264.7 macrophages (5), but whether the further elevation of CD14 in the absence of CD9 occurs at the mRNA or protein level remains to be investigated. Second, larger portions of CD14 and TLR4 proteins were concentrated into light membrane fractions relative to dense fractions in CD9 KO BMDMs than in WT cells. CD9 is an abundantly expressed tetraspan in macrophages (29) and, in WT BMDMs, the population of CD9 molecules recruited into the lipid-rich microdomains would not be small. So, the absence of CD9 might remove its direct interference with CD14 signaling complexes at the microdomains. These alterations most likely increase functional CD14/TLR4 receptor clusters, the composition and stoichiometry of which are important determinants for immune response (20). Thus, we speculate that CD9 KO BMDMs are in “primed” conditions and, upon LPS binding, they are easily and rapidly activated. The platform where CD9 regulates the LPS response is a cholesterol-enriched microdomain, and this is essential for the CD9 function because the level of TNF-α production of CD9 KO BMDMs was reduced to the same level as WT cells after cholesterol depletion with 2OHpCD (Fig. 6). Collectively, it seems that macrophage CD9 negatively regulates the LPS response by preventing CD14-mediated signaling at lipid-enriched microdomains.

Among other signaling molecules tested, the expression of SIRPs appeared slightly lower in CD9 KO BMDMs (Fig. 5). A recent report showed that the SIRPs level of macrophages is important to regulate LPS signaling negatively at an early stage of TLR4 activation (30). Although detailed mechanisms remain to be studied, there is a possibility that the decreased SIRPα may also participate in CD9-mediated negative regulation of LPS response. CD81 is another macrophage tetraspanin closely related to CD9 (7). This tetraspanin showed the redistribution similar to CD9 in sucrose gradients; a portion of CD81 protein was concentrated to light membrane fractions upon LPS stimulation (Fig. 4). Interestingly, in CD9 KO BMDMs, CD81 distribution was shifted to light fractions compared with WT cells (Fig. 4). Possibly, this might mean CD81 compensates for the loss of CD9 function at lipid microdomains as proposed previously (7). Using a fluorescence resonance energy transfer technique, participation of CD81 into the CD14/TLR4 receptor clustering at the lipid membrane microdomain has been reported in previous studies (15, 3, 20). These studies displayed that stimulation of human monocytes with LPS induced coassembly of CD14, TLR4, CD11b/CD18, CD81, and other molecules in vitro, although the meaning of the CD81 participation remained unknown. They further provided data suggesting a coassembly of CD81 and CD14 in monocytes from patients with sepsis (i.e., in vivo LPS stimulation) (15). CD9 was also examined in their recent study (28) but, unlike the present study, CD9 appeared not to be associated with the lipid microdomain in a rapid detergent-based flow cytometric assay. This discrepancy may be due to methodological differences or might be because they used human monocytes from healthy donors (i.e., without in vivo LPS stimulation) in this CD9 experiment (28). Studies using CD81 KO macrophages and, hopefully, CD9/CD81 double-KO macrophages, will be needed to delineate coordination of tetraspanins CD9 and CD81 in the LPS-induced CD14 signaling.
In conclusion, we have shown that LPS-induced lung inflammation is exacerbated in the absence of CD9 function. As part of its mechanisms, we propose that CD9 negatively regulates LPS-induced macrophage activation by preventing CD14-dependent receptor assembly at lipid-enriched membrane microdomains. LPS is one of molecules contained by cigarette smoke that leads to pulmonary emphysema, and also causes fatal sepsis syndrome including acute lung injury in humans. Thus, the deficiency of CD9 function may be an important predisposing factor to such inflammatory lung diseases.

Acknowledgments
We thank Dr. E. Mekada for generously providing CD9 KO mice and helpful comments on this paper, and Y. Habe for secretarial assistance.

Disclosures
The authors have no financial conflict of interest.

References