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Monocytic Thrombomodulin Triggers LPS- and Gram-Negative Bacteria-Induced Inflammatory Response

Chih-Yuan Ma,*‡ Guey-Yueh Shi,*† Chung-Sheng Shi,*‡ Yuan-Chung Kao,*‡ Shu-Wha Lin,*† and Hua-Lin Wu,*‡†

Sepsis results from the host hyperinflammatory response to bacterial infection, causing multiple organ failure and high mortality. We previously demonstrated that LPS binds to monocytic membrane-bound thrombomodulin (TM), but the role of monocytic TM in LPS-induced inflammation remains unknown. In this study, we demonstrated that TM knockdown in human monocytic cells attenuated LPS-induced signaling pathways and cytokine production. Coimmunoprecipitation and immunofluorescence assays showed that monocytic TM interacted with the LPS receptors, CD14 and TLR4/myeloid differentiation factor-2 (MD-2) complex, indicating that it binds to LPS and triggers an LPS-induced inflammatory response by interacting with the CD14/TLR4/MD-2 complex. We also found that monocytic TM knockdown reduced cytokine production induced by Gram-negative bacteria Klebsiella pneumoniae, suggesting that monocytic TM plays an important role in Gram-negative bacteria-induced inflammation. To further investigate the function of monocytic TM in vivo, myeloid-specific TM-deficient mice were established and were found to display improved survival that resulted from the attenuation of septic syndrome, including reduced systemic inflammatory response and resistance to bacterial dissemination, after K. pneumoniae infection or cecal ligation and puncture surgery. The inhibition of bacterial dissemination in mice with a deficiency of myeloid TM may be caused by the early increase in neutrophil infiltration. Therefore, we conclude that monocytic TM is a novel component in the CD14/TLR4/MD-2 complex and participates in the LPS- and Gram-negative bacteria-induced inflammatory response.

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A host’s hyperinflammatory response to a bacterial infection leads to severe sepsis, resulting in multiple organ failure and high mortality (1–3). An inflammatory response in a host is caused primarily by cytokines, such as TNF-α, IL-1β, and IL-6, which are produced by monocytes and macrophages after the recognition of pathogen-associated molecular patterns by pattern-recognition receptors (4, 5). The cell wall component of Gram-negative bacteria LPS is frequently used to mimic clinical septic syndrome by inducing a hyperinflammatory response in hosts (1–3). Reports indicated that the LPS-binding protein binds to LPS in the plasma and immediately transfers LPS to CD14 on the monocytic cells (6, 7). CD14 subsequently interacts with TLR4 and activates intracellular signaling pathways, including the MAPK and NF-κB pathways (5, 8, 9). Activation of these signaling pathways in monocytes and macrophages can result in the production of inflammatory cytokines (5).

Thrombomodulin (TM) is a type I transmembrane glycoprotein, consisting of five domains: a C-type lectin-like domain, an epidermal growth factor (EGF)-like domain, a serine/threonine-rich domain, a transmembrane domain, and a cytoplasmic domain (10). TM was originally identified in endothelial cells and functions as an anticoagulant factor through interaction with thrombin. The TM–thrombin complex efficiently activates protein C to activated protein C, which enzymatically degrades factors Va and VIIIa to terminate blood coagulation (11, 12). TM may regulate embryonic development, because whole-body TM knockout in mice leads to embryonic lethality (13). Recent research showed that mice with TM lectin-like domain deletion have a greater inflammatory response to LPS than do wild-type (WT) mice (14), indicating that the TM lectin-like domain may regulate host inflammatory response to LPS. In addition, our earlier studies showed that TM overexpression in melanoma cells efficiently increases cell–cell adhesion and that TM knockdown in tumor cells facilitates cell migration and proliferation (15, 16). Soluble forms of TM are also found in human plasma and urine (17). We previously demonstrated that the combination of a recombinant TM EGF-like domain with a serine/threonine-rich domain can be a novel angiogenic factor for promoting endothelial cell proliferation and migration (18). Furthermore, recombinant TM lectin-like domain binds to the Lewis Y Ag on LPS and neutralizes the LPS-induced inflammatory response (19). These data suggest that TM not only participates in the regulation of anticoagulation but also has multiple functions in physiology and pathology.

TM expression in monocyes and macrophages has been identified (20), but the physiological function of monocytic TM is...
poorly understood. Previous studies mostly focused on monocytic TM expression in response to LPS (20–22). However, TM expression was downregulated in human PBMCs or primary monocytes (20, 22), whereas the upregulation of TM expression was found in human monocytic THP-1 cells after LPS stimulation (21). In this study, we used an RNA interference technique and tissue-specific knockout mice to investigate the role of monocytic TM in LPS- and Gram-negative bacteria-induced inflammation in vitro and in vivo.

Materials and Methods

Cell culture

Human monocytic THP-1 and U-937 cells (Bioresource Collection and Research Center, Taiwan) were maintained in RPMI 1640 medium supplemented with 10% FBS at 37°C. For isolation of mouse peritoneal macrophages, mice (8–12 wk of age) were injected i.p. with 4% thioglycolate. After 4 d, mice were sacrificed, and peritoneal exudates were obtained by peritoneal lavage with PBS. For purification of macrophages, peritoneal exudate cells were incubated in culture dishes with RPMI 1640 medium containing 10% FBS and 1% penicillin/streptomycin (v/v). After isolation of mouse peritoneal macrophages, mAbs (8–12 wk of age) were injected i.p. with 4% thioglycolate. After 4 d, mice were sacrificed, and peritoneal exudates were obtained by peritoneal lavage with PBS.

Biotinylated LPS pull-down assay

For unlabeled LPS competition, THP-1 cells were coincubated with biotinylated LPS (Escherichia coli O111:B4; InvivoGen) and unlabeled LPS (E. coli O111:B4; Sigma-Aldrich) for 30 min at 37°C. After incubation, cells lysates were harvested and incubated with streptavidin-conjugated agarose resin (Thermo Scientific) for 60 min at 4°C. Resins were washed three times with PBS containing 0.05% Tween-20 and analyzed by Western blotting. Mouse anti-human TM mAb (D3 clone) was purchased from Santa Cruz Biotechnology. For recombinant TM lectin-like domain, recombinant human TM lectin-like domain was prepared as described above. The TM flox/+ mice were bred with WT C57BL/6 mice for at least five generations. The LysMcre, in which Cre recombinase was driven by the LysM promoter, was used as a control, and the mouse anti–α-tubulin mAb was purchased from Abcam.

Generation of myeloid-specific TM-deficient mice (LysMcre/ TMflox/flox mice)

A targeting vector containing a 12.3-kb fragment (5’ arm of homology) and a 5-kb fragment (3’ arm of homology) was constructed. The only exon of the TM gene was floxed (flanked by loxP sites) and removed in the presence of Cre recombinase. The targeting vector was linearized and electroporated into the R1 hybrid embryonic stem cells of mixed 129X1/SvJ and 129S1 background. To remove the selection cassette, Fpl recombination was transiently expressed in targeted embryonic stem cells that were injected into C57BL/6 blastocysts, and embryos were implanted into pseudopregnant CD-1 foster mothers. Sequencing, Southern blotting, and generation of TMflox/+ chimera mice were performed by the Transgenic Mouse Models Core, National Research Program for Genomic Medicine. The TMflox/+ mice were bred with WT C57BL/6 mice for at least five generations. The LysMcre mice, in which Cre recombinase was driven by

Statistical analysis was performed by an unpaired t test. *p < 0.05, **p < 0.01.
lysozyme M promoter and was mainly expressed in myeloid lineage cells (23), were obtained from The Jackson Laboratory and crossed with TM_DN mice to generate myeloid-specific TM-deficient mice. The TM_DN mice and LysMcre/TM_DN mice littermates were used in further experiments. For genotyping, genomic DNA was extracted from mouse tail and analyzed by PCR. The forward primer sequence of cre was 5'-CTTG-GGCTGCCGAAATTCTC-3' and the reverse primer sequence of cre was 5'-CCCAGAAATGCGCAGATTACG-3'. The forward primer of loxP sequence was 5'-AAAGCTTGGTGTCATTTC-3' and the reverse primer sequence of loxP was 5'-GTGAAATGACGGCCGACAT-3'. Because commercial Abs against mouse TM are not efficient for mouse TM analysis, we prepared a recombinant mouse TM EGF-like domain combined with serine/threonine-rich domain as an immunogen. After rabbit immunization, the rabbit anti-mouse TM polyclonal Ab in the sera was purified by CNBr-activated Sepharose (GE Healthcare) coupled with the recombinant mouse TM EGF-like domain combined with the serine/threonine-rich domain. The purified rabbit anti-mouse TM polyclonal Ab was used in Western blotting and immunofluorescence assay.

For analysis of Cre recombinase specificity in LysMcre/TM_DN mice, global double-fluorescent Cre reporter mice (mTmG mice) (24) were used (The Jackson Laboratory). mTmG mice were crossed with TM_DN mice or LysMcre/TM_DN mice to generate mTmG/TM_DN and mTmG/ LysMcre/TM_DN mice. Tissues were perfused with PBS and isolated from sacrificed mice, immersed in 20% sucrose/PBS for 24 h at 4°C, and embedded in OCT compound overnight at room temperature (RT). Frozen sections (5 µm/section) were obtained, and images were taken using a Leica fluorescence microscope.

Coimmunoprecipitation and immunofluorescence assays

For the coimmunoprecipitation assay, mouse peritoneal macrophages were treated or not with LPS (100 ng/ml) for 30 min at 37°C. Cell lysates were harvested and incubated with rabbit anti-mouse CD14 polyclonal Ab (Abnova), rabbit anti-mouse TLR4 polyclonal Ab (Abnova), or rabbit anti-mouse myeloid differentiation factor-2 (MD-2) polyclonal Ab (Abcam). Immune complexes were precipitated by protein A agarose beads over-
night at 4°C. Rabbit IgG Ab served as a negative control. beads were washed thrice with PBS containing 0.05% Tween-20 and analyzed by Western blotting. Rabbit anti-mouse TLR4 polyclonal Ab (Cell Signaling Technology) was used to detect the TLR4 signal in Western blotting.

For the immunofluorescence assay, mouse peritoneal macrophages were seeded onto coverslips overnight at 37°C. Cells were treated with biotinylated LPS (5 μg/ml) for 30 min at 37°C, fixed with 3.7% formaldehyde/PBS for 10 min at 37°C, permeabilized with 0.2% Triton X-100/PBS for 1 min at RT, blocked with 10% PBS/PBS for 30 min at RT, incubated with primary Abs for 60 min at RT, and stained by fluorescent streptavidin and secondary Abs for 60 min at RT. Three groups of triple stain were performed and the pair of Abs was described as follows: rat anti-mouse CD14 mAb (BioLegend) and rabbit anti-mouse TLR4 polyclonal Ab (Abnova); rat anti-mouse CD14 mAb (BioLegend) and rabbit anti-mouse TM polyclonal Ab; and rat anti-mouse TLR4 mAb (IMGENEX) and rabbit anti-mouse TM polyclonal Ab. Alexa Fluor 647-conjugated streptavidin (Invitrogen) was used to stain the biotinylated LPS. Alexa Fluor 488-conjugated goat anti-rat Ab (Invitrogen) and Alexa Fluor 564-conjugated goat anti-rabbit Ab (Invitrogen) were used in these three groups. All images were acquired using an Olympus confocal microscope.

Flow cytometry

For LPS-binding assay, human monocytic THP-1 cells were incubated with FITC-LPS (Sigma-Aldrich) and recombinant TM lectin-like domain for 30 min at 4°C. Cells were washed thrice with ice-cold PBS and further analyzed by FACS (BD Biosciences). Mouse peritoneal macrophages were incubated with PBS or FITC-LPS (50 μg/ml) for 30 min at 4°C. Cells were washed thrice with ice-cold PBS and further analyzed by FACS (BD Biosciences). Geometric mean fluorescence intensity (MFI) was measured using WinMDI 2.92 software.

For analyzing TM expression in blood monocytes, mouse blood was collected by cardiac puncture, anduffy coats were obtained by ficoll-paque density-gradient centrifugation. cells were stained with FITC-conjugated rat anti-mouse CD14 mAb (monocyte surface marker; eBioscience) and PE-conjugated rat anti-mouse TM mAb (R&D systems). The TM expression of CD14+ cells was analyzed by FACS. For analyzing TM expression in neutrophils, neutrophils were obtained from the peritoneum 24 h after administering thioglycollate. Cells were stained with FITC-conjugated rat anti-mouse Gr-1 mAb (neutrophil surface marker; eBioscience) and PE-conjugated rat anti-mouse TM mAb. TM expression of Gr-1+ cells was analyzed by FACS.

For analyzing neutrophil infiltration into the peritoneum, mice were injected i.p. with K. pneumoniae, or cecal ligation and puncture (CLP) was performed. Four hours later, the peritoneal fluid was harvested, and the cells were stained with FITC-conjugated rat anti-mouse Gr-1 mAb. The percentage of Gr-1+ cells was determined by FACS.

Experimental sepsis models

For the bacteremia model, a high dose of K. pneumoniae (1 × 10⁸ CFU/mouse), a low dose of K. pneumoniae (1 × 10⁷ CFU/mouse), or S. aureus (1 × 10⁷ or 1 × 10⁸ CFU/mouse) were injected i.p. into mice (8–12 wk of age). After stimulation, sera were harvested at 6 and 12 h for the determination of TNF-α and IL-6 concentration by ELISA. Mouse survival was observed every 24 h. For analysis of lung injury, mice received the high-grade CLP procedure and were sacrificed at 12 h post-surgery. Frozen sections of the lung (5 μm/section) were obtained, as described above, and stained with H&E. All images were acquired using a Leica light microscope. All animal experiments were approved by The Institutional Animal Care and Use Committee of National Cheng Kung University.

Statistical analyses

Statistical significance was analyzed using a parametric unpaired t test, and differences between more than two groups were compared using two-way ANOVA with a Bonferroni posttest. Survival data were analyzed using a log-rank test. The p values < 0.05 were considered statistically significant.

Results

LPS binds to the lectin-like domain of the monocytic membrane-bound TM

The interaction between LPS and monocytic membrane-bound TM was demonstrated in an earlier study using a biotinylated LPS pull-down assay (19). To further clarify the specificity of this interaction, unlabeled LPS was used to compete with the binding of biotinylated LPS to monocytic membrane-bound TM. As shown in Fig. 1A, this finding is consistent with an earlier observation that monocytic membrane-bound TM could be pulled down by biotinylated LPS. In addition, unlabeled LPS dose dependently competed with the binding of biotinylated LPS to monocytic membrane-bound TM (Fig. 1A), suggesting that LPS specifically binds to monocytic membrane-bound TM. We also found that the interaction between biotinylated LPS and monocytic membrane-bound TM was decreased by recombinant TM lectin-like domain in a dose-dependent manner (Fig. 1B), and recombinant TM lectin-like domain could reduce the binding of FITC-LPS to monocytic cell membrane (Fig. 1C). These data suggest that LPS binds to the lectin-like domain of monocytic membrane-bound TM.

TM knockdown in human monocytic cells suppresses LPS-induced signaling pathways and cytokine production

To analyze the role of monocytic TM in the LPS-induced inflammatory response, we knocked down TM expression in human monocytic THP-1 and U-937 cells with lentivirus-delivered shTM-L and measured LPS-induced cytokine production. TM expression was greatly downregulated by shTM-L in THP-1 (Fig. 2A) and U-937 cells (Fig. 2B). Production of inflammatory cytokines, including TNF-α, IL-1β, and IL-6, was suppressed in TM knock-
down THP-1 and U-937 cells compared with control cells after LPS stimulation (Fig. 2A, 2B). A similar result was obtained in TM knockdown human PBMCs, which produced lower TNF-α concentrations than did the control cells (Fig. 2C). These results suggest that monocytic TM may participate in promoting the inflammatory response to LPS.

To exclude off-target effects, another targeting sequence, shTM-2, was used to knockdown TM expression in U-937 cells (Fig. 2D). The results showed that TM expression and LPS-induced IL-6 production were suppressed in TM knockdown (shTM-2) U-937 cells and that IL-6 concentration was positively correlated with monocytic TM expression levels (Fig. 2D). Moreover, RR-TM not only rescued TM expression in TM knockdown (shTM-1) U-937 cells, it also restored IL-6 production after LPS treatment (Fig. 2E), indicating that monocytic TM knockdown results in decreased production of inflammatory cytokines in response to LPS.

We also demonstrated that both ERK1/2 phosphorylation and IκB-α degradation were attenuated in TM knockdown (shTM-1) THP-1 cells at 60 min after LPS stimulation (Fig. 2F), and densitometry of pERK1/2 was performed (Supplemental Fig. 1). These results reveal that monocytic TM may bind to LPS and activate LPS-induced signaling pathways to produce inflammatory cytokines.

**Monocytic TM knockdown specifically reduces LPS- and Gram-negative bacteria-induced cytokine production**

To further analyze the role of monocytic TM in the inflammatory response to different bacteria and bacterial components, TM knockdown (shTM-1) THP-1 cells were stimulated with PBS, LPS, LTA, heat-killed Gram-negative bacteria (*K. pneumoniae*), or heat-killed Gram-positive bacteria (*S. aureus*) for 24 h at 37°C. As shown in Fig. 3, upon stimulation with only LPS and *K. pneumoniae*, TNF-α production was suppressed in TM knockdown (shTM-1) THP-1 cells, whereas monocytic TM knockdown did not affect TNF-α levels in the medium after stimulation with LTA. Furthermore, TNF-α concentration was increased by *S. aureus* in

**FIGURE 4.** LysMcre/TM<sup>flx/flx</sup> mice established by the Cre/loxP system and TM knockout macrophages produce lower levels of TNF-α in response to LPS and *K. pneumoniae* stimulation. (A) Schematic illustration of the floxed TM gene. (B) TM<sup>flx/flx</sup> and LysMcre/TM<sup>flx/flx</sup> mice were generated by the Cre/loxP system, and their genotypes were analyzed by PCR. (C) Peritoneal macrophages were obtained from TM<sup>flx/flx</sup> and LysMcre/TM<sup>flx/flx</sup> mice, and the cell lysates were harvested for analysis of TM expression by Western blotting. (D) Tissues were harvested from mTmG/TM<sup>flx/flx</sup> and mTmG/LysMcre/TM<sup>flx/flx</sup> mice, and frozen sections (5 μm/section) were obtained for image acquisition (original magnification ×200 for peritoneal macrophages, ×100 for other tissues). All images were acquired using a Leica fluorescence microscope. (E) Peritoneal macrophages obtained from TM<sup>flx/flx</sup> and LysMcre/TM<sup>flx/flx</sup> mice were stimulated with PBS, LPS (100 ng/ml), LTA (5 μg/ml), heat-killed *K. pneumoniae* (1 × 10<sup>7</sup> CFU/well), or heat-killed *S. aureus* (1 × 10<sup>7</sup> CFU or 5 × 10<sup>7</sup> CFU/well) for 6 h at 37°C. The culture media were harvested for the determination of TNF-α concentration by ELISA. Data shown are mean ± SEM (n = 3). *p < 0.05, **p < 0.01, two-way ANOVA with a Bonferroni posttest.
a dose-dependent manner, and TM knockdown did not interfere with the TNF-α production induced by S. aureus. This result suggests that monocytic TM plays a crucial role in LPS- and K. pneumoniae-induced inflammation.

Establishment of myeloid-specific TM-deficient mice

To investigate the physiological function of monocytic TM in vivo, myeloid-specific TM-deficient mice, LysMcre/TMfloxfloxflox mice, were generated using the Cre-loxP system. A schematic diagram of floxed TM gene is shown in Fig. 4A. No defects in the LysMcre/TMfloxfloxflox mice were observed, including the number of littermates, body weight, and other postnatal phenotypes. The genotypes of the TMfloxfloxflox mice and the LysMcre/TMfloxfloxflox mice were confirmed by PCR (Fig. 4B); Western blotting showed that TM expression was undetectable in peritoneal macrophages obtained from LysMcre/TMfloxfloxflox mice (Fig. 4C). To further confirm that Cre recombinase is mainly expressed in myeloid lineage cells of LysMcre/TMfloxfloxflox mice, we used global double-fluorescent Cre reporter mice (mTmG mice) (24). The gene structure of mTmG mice was designed as loxP-mT (membrane-targeted tandem dimer Tomato)-loxP-polyadenylation-mG (membrane-targeted enhanced GFP [EGFP])-polyadenylation. When Cre recombinase was expressed in the cells, the red fluorescence was blocked by Cre recombinase excision, and EGFP was expressed in the cells. Multiple tissues, including those of the peritoneal macrophages, heart, lung, liver, spleen, and kidney, were isolated from mTmG/
TM^flox/flox^ mice and mTmG/LysMcre/TM^flox/flox^ mice for analysis of Cre recombinase specificity in LysMcre/TM^flox/flox^ mice. As shown in Fig. 4D, EGFP expression was detected in peritoneal macrophages obtained from mTmG/LysMcre/TM^flox/flox^ mice but not in other tissues obtained from either mTmG/TM^flox/flox^ or mTmG/LysMcre/TM^flox/flox^ mice. TM expression in monocytes and neutrophils was suppressed in LysMcre/TM^flox/flox^ mice (Supplemental Fig. 2A, 2B), suggesting that Cre recombinase is primarily expressed in the myeloid lineage cells of LysMcre/TM^flox/flox^ mice.

To further prove that monocytic TM plays a role in LPS- and K. pneumoniae-induced inflammation, peritoneal macrophages isolated from TM^flox/flox^ mice and LysMcre/TM^flox/flox^ mice were treated with different bacteria and bacterial components, and the TNF-α concentration was measured. As shown in Fig. 4E, peritoneal macrophages from LysMcre/TM^flox/flox^ mice had a decreased response to LPS and K. pneumoniae compared with those of TM^flox/flox^ mice, whereas no significant difference was observed between the two groups of macrophages in response to LTA and S. aureus. This result, together with the data obtained from TM knockdown in human monocytic cells (Fig. 3), suggests that monocytic TM is involved in the LPS- and K. pneumoniae-induced inflammatory response.

Monocytic TM interacts with the CD14/TLR4/MD-2 complex and facilitates LPS binding to cell surfaces

LPS stimulation increases CD14/TLR4 complex formation in monocytic cells, leading to intracellular-signaling pathway activation and inflammatory cytokine production (8, 9). Because inflammatory cytokine production was greatly suppressed in TM-deficient monocytic cells in response to LPS (Figs. 2, 4E), we proposed that TM might interact with CD14 or TLR4 during LPS stimulation. We also analyzed the interaction between CD14 and TLR4 using a coimmunoprecipitation assay; the results were consistent with those of earlier reports (8, 9) stating that CD14 forms a complex with TLR4 after LPS stimulation. MD-2 is a critical component of the TLR4 complex involved in the LPS response and TLR4 distribution (26). The interaction between TM and MD-2 was investigated by a coimmunoprecipitation assay (Fig. 5C). Colocalization of LPS, TM, CD14, and TLR4 was also observed on the plasma membrane of macrophages through use of an immunofluorescence assay (Fig. 5D). Notably, TM knockout in macrophages did not interfere with CD14/TLR4 complex formation (Fig. 5B), indicating that TM may be an accessory protein in this complex. We also found that LPS binding to the cell surface

**FIGURE 6.** LysMcre/TM^flox/flox^ mice exhibit improved survival rate and suppression of septic syndrome after K. pneumoniae infection. (A) Mouse survival was monitored every 24 h after infection with a low dose of K. pneumoniae (1 × 10^3 CFU/mouse) or a high dose of K. pneumoniae (1 × 10^5 CFU/mouse) (n = 6–13 mice/group). (B) Leukocyte infiltration into the lung was analyzed by histological staining with H&E 12 h after infection with a high dose of K. pneumoniae (1 × 10^5 CFU/mouse). Original magnification ×100. Sera were harvested at 6 and 12 h after infection with K. pneumoniae (low dose, 1 × 10^3 CFU per mouse; high dose, 1 × 10^5 CFU/mouse; n = 6–8 mice/group) or S. aureus (1 × 10^3 or 1 × 10^5 CFU/mouse; n = 3–5 mice/group) (C, D) or for the determination of TNF-α and IL-6 concentrations, respectively, by ELISA. (E) Blood was harvested from mice 12 h after infection with K. pneumoniae (low dose, 1 × 10^3 CFU/mouse; high dose, 1 × 10^5 CFU/mouse), cultured on Luria–Bertani agar (n = 5–6 mice/group), and the colonies of K. pneumoniae were counted. Data shown are mean ± SEM and are representative of three independent experiments. Statistical differences between the survival of TM^flox/flox^ and LysMcre/TM^flox/flox^ mice were analyzed using the log-rank test, and the analyses of cytokine concentration and CFU were performed using an unpaired t test. *p < 0.05, **p < 0.01.
was significantly decreased in TM knockout macrophages (Fig. 5E), suggesting that monocyctic TM facilitates binding of LPS to the cell surface and elicits the LPS-induced inflammatory response by interacting with the CD14/TLR4/MD-2 complex.

**Improved survival and reduced septic syndrome are observed in LysMcre/TM<sup>flx/flx</sup> mice after K. pneumoniae infection or CLP surgery**

We next investigated the role of myeloid TM in <i>K. pneumoniae</i>-induced bacteremia in vivo. As shown in Fig. 6A, all TM<sup>flx/flx</sup> mice died 24 h after infection with a high dose of <i>K. pneumoniae</i> (1 x 10<sup>5</sup> CFU/mouse), whereas similarly infected LysMcre/TM<sup>flx/flx</sup> mice had a 50% survival rate at 24 h. However, all LysMcre/TM<sup>flx/flx</sup> mice succumbed to infection at 72 h after infection with a high dose of <i>K. pneumoniae</i>. In addition, after infection with a low dose of <i>K. pneumoniae</i> (1 x 10<sup>3</sup> CFU/mouse), all TM<sup>flx/flx</sup> mice died at 96 h, and 33% of LysMcre/TM<sup>flx/flx</sup> mice subjected to high-grade CLP died at 24 h, but 21% survival was observed in LysMcre/TM<sup>flx/flx</sup> mice at 168 h (Fig. 6B). Midgrade CLP was also performed in TM<sup>flx/flx</sup> mice and LysMcre/TM<sup>flx/flx</sup> mice, and all TM<sup>flx/flx</sup> mice died at 72 h, whereas 43% of LysMcre/TM<sup>flx/flx</sup> mice survived for >168 h (Fig. 7A). Reduced lung injury in LysMcre/TM<sup>flx/flx</sup> mice was also observed at 12 h after infection with a high dose of <i>K. pneumoniae</i> (1 x 10<sup>5</sup> CFU/mouse) (Fig. 6B) and 12 h after high-grade CLP (Fig. 7B). Furthermore, the concentrations of both TNF-α and IL-6 in sera were significantly decreased in LysMcre/TM<sup>flx/flx</sup> mice after infection with a low dose (1 x 10<sup>3</sup> CFU/mouse) or a high dose (1 x 10<sup>5</sup> CFU/mouse) of <i>K. pneumoniae</i> (Fig. 6C, 6D), and the serum IL-6 concentration was also reduced in LysMcre/TM<sup>flx/flx</sup> mice after midgrade or high-grade CLP (Fig. 7C). <i>S. aureus</i>-induced IL-6 secretion was also analyzed in TM<sup>flx/flx</sup> mice and LysMcre/TM<sup>flx/flx</sup> mice. Serum IL-6 concentration was very low in the PBS group and was induced by <i>S. aureus</i> infection in TM<sup>flx/flx</sup> mice and LysMcre/TM<sup>flx/flx</sup> mice in a dose-dependent manner. However, there was no significant difference in serum IL-6 concentration between the two groups of mice after infection with <i>S. aureus</i> (1 x 10<sup>3</sup> or 1 x 10<sup>5</sup> CFU/mouse) (Fig. 6F). These results indicate that suppression of the systemic inflammatory response to <i>K. pneumoniae</i> infection and CLP may result from poor recognition of LPS in LysMcre/TM<sup>flx/flx</sup> mice.

We also measured the bacterial CFU in the blood of TM<sup>flx/flx</sup> and LysMcre/TM<sup>flx/flx</sup> mice after infection with a low dose or a high dose of <i>K. pneumoniae</i> (Fig. 6E) and midgrade or high-grade CLP surgery (Fig. 7D). In both conditions, loss of myeloid TM could significantly reduce bacterial dissemination in mice. This result is in accordance with previous observations that mice with whole-body knockout of CD14 exhibit lower inflammatory response to <i>K. pneumoniae</i> infection and CLP surgery. Mice were injected i.p. with <i>K. pneumoniae</i> (1 x 10<sup>5</sup> CFU/mouse; n = 7 mice/group) (A) or were subjected to midgrade CLP (n = 5–14 mice/group) (B). Four hours later, peritoneal fluid was harvested, and the cells were stained with FITC-conjugated rat anti-mouse Gr-1 Ab. The percentage of Gr-1<sup>+</sup> cells was determined by FACS. Data shown are mean ± SEM and are representative of three independent experiments. *p < 0.05, unpaired t test.

**FIGURE 7.** Reduced septic syndrome is observed in LysMcre/TM<sup>flx/flx</sup> mice after CLP surgery. (A) Midgrade or high-grade CLP surgery was performed in mice, and survival was monitored every 24 h (n = 5–14 mice/group). (B) Leukocyte infiltration into the lung was analyzed by histological staining with H&E 12 h after high-grade CLP surgery. Original magnification ×100. (C) After 12 h, serum IL-6 concentration was determined by ELISA (n = 5–14 mice/group), and blood was cultured on Luria–Bertani agar. (D) The colonies of bacteria on Luria–Bertani agar plates were counted (n = 6–7 mice/group). Data shown are mean ± SEM and are representative of three independent experiments. Statistical differences between the survival of TM<sup>flx/flx</sup> and LysMcre/TM<sup>flx/flx</sup> mice were analyzed using the log-rank test, and IL-6 concentration and CFU analyses were performed using an unpaired t test. *p < 0.05.

**FIGURE 8.** LysMcre/TM<sup>flx/flx</sup> mice exhibit increased neutrophil infiltration into the peritoneum at the early stages of <i>K. pneumoniae</i> infection and CLP surgery. Mice were injected i.p. with <i>K. pneumoniae</i> (1 x 10<sup>5</sup> CFU/mouse; n = 7 mice/group) (A) or were subjected to midgrade CLP (n = 5–14 mice/group) (B). Four hours later, peritoneal fluid was harvested, and the cells were stained with FITC-conjugated rat anti-mouse Gr-1 Ab. The percentage of Gr-1<sup>+</sup> cells was determined by FACS. Data shown are mean ± SEM and are representative of three independent experiments. *p < 0.05, unpaired t test.
cytokine secretion resulting from poor recognition of LPS, as well as suppression of Gram-negative bacterial dissemination caused by early neutrophil infiltration (27, 28). Therefore, we tested the infiltration of neutrophils into the peritoneum of TMfloxflox mice and LysMcre/TMfloxflox mice after K. pneumoniae infection or CLP surgery. As shown in Fig. 8, the infiltration of neutrophils in LysMcre/TMfloxflox mice was significantly increased 4 h after infection with a high dose of K. pneumoniae (1 × 10⁵ CFU/mouse) (Fig. 8A) or midgrade CLP surgery (Fig. 8B) compared with TMfloxflox mice, indicating that the reduced bacterial dissemination in LysMcre/TMfloxflox mice may be caused by this early increase in neutrophil infiltration.

These data suggest that monocytic TM is involved in the host inflammatory response to bacterial infection, especially to Gram-negative bacteria (such as K. pneumoniae). After K. pneumoniae infection or CLP surgery, mice lacking TM in myeloid cells exhibit improved survival, resulting from the attenuation of septic syndrome, including suppression of systemic inflammation caused by poor recognition of LPS and resistance to bacterial dissemination caused by the early increase in neutrophil infiltration.

Discussion

Our earlier research showed that both recombinant TM lectin-like domain and monocytic membrane-bound TM bind to LPS (19), but the function of monocytic TM in LPS-induced inflammation remains unknown. In this study, we further confirmed that LPS specifically binds to the lectin-like domain of monocytic membrane-bound TM and that TM knockdown in human monocytic cells attenuated LPS- and Gram-negative bacteria-induced inflammatory response. We also demonstrated that monocytic TM facilitated LPS binding to cell surfaces and triggered an LPS-induced inflammatory response by interacting with the CD14/TLR4/MD-2 complex. Myeloid TM knockout mice had reduced septic syndrome and increased survival after K. pneumoniae infection or CLP surgery. This study demonstrates that monocytic TM is one of the components of the CD14/TLR4/MD-2 complex and that it participates in promoting the inflammatory response to LPS and Gram-negative bacteria.

Although TM is a type I transmembrane glycoprotein, soluble TM has been found in human plasma and urine (17). The levels of soluble TM in plasma are drastically increased in patients with sepsis and organ failure (29). It was reported that mice with TM lectin-like domain deletion show greater inflammatory response to bacterial infection, especially to Gram-negative bacterial dissemination in LysMcre/TMfloxflox mice after K. pneumoniae infection (Fig. 6E) or CLP surgery (Fig. 7D). This finding is in accordance with the previous observations that CD14-deficient mice exhibit reduced inflammatory response to Gram-negative bacteria, as well as suppression of Gram-negative bacterial dissemination (27). Additional evidence shows that the inhibition of bacterial dissemination in CD14-deficient mice is due to early neutrophil infiltration (28). Our present data also demonstrated that infiltration of neutrophils into the peritoneum was significantly increased in LysMcre/TMfloxflox mice compared with TMfloxflox mice at the early stages of K. pneumoniae infection or CLP surgery (Fig. 8), suggesting that suppression of bacterial dissemination in LysMcre/TMfloxflox mice may result from this early increase in neutrophil infiltration.

In conclusion, the role of monocytic TM is to participate in the inflammatory response to LPS by binding the LPS to cell surfaces and interacting with the CD14/TLR4/MD-2 complex that may induce a hyperinflammatory host response to Gram-negative bacterial infection. Loss of monocytic TM in mice can effectively reduce the hyperinflammatory response to bacterial infection. Furthermore, myeloid TM deficiency in mice leads to an early increase in neutrophil infiltration that effectively inhibits bacterial dissemination after K. pneumoniae infection or CLP surgery.

Disclosures

The authors have no financial conflicts of interest.

References

Supplemental Figure 1. Densitometry of pERK1/2 in TM-knockdown THP-1 cells after LPS stimulation. TM-knockdown THP-1 cells were stimulated with LPS (100 ng/ml) for the indicated time points. Cell lysates were harvested and analyzed by Western blotting. Relative intensity was obtained from the normalization of the densitometry of pERK1/2 with the densitometry of α-tubulin. Data shown are expressed as mean ± SEM, n = 3. Statistical analysis was performed by 2-way ANOVA with a Bonferroni post-test. *, p < 0.05.
Supplemental Figure 2. TM expression of monocytes and neutrophils in LysMcre/TM\textsuperscript{floxflox} mice is suppressed by Cre recombinase expression. A, Mouse blood was collected by cardiac puncture, and buffy coats were obtained by Ficoll-Paque density gradient centrifugation. Cells were stained with...
FITC-conjugated rat anti-mouse CD14 antibody and PE-conjugated rat anti-mouse TM antibody. TM expression of CD14$^+$ cells was analyzed by FACS. B, Neutrophils were obtained and stained with FITC-conjugated rat anti-mouse Gr-1 antibody and PE-conjugated rat anti-mouse TM antibody. TM expression of Gr-1$^+$ cells was analyzed by FACS.