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*J Immunol* 2010; 184:5160-5171; Prepublished online 24 March 2010; doi: 10.4049/jimmunol.0902663

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Suppression of Host Innate Immune Response by *Burkholderia pseudomallei* through the Virulence Factor TssM

Kai Soo Tan,* Yahua Chen,* Yaw-Chyn Lim,†‡§ Gek-Yen Gladys Tan,* Yichun Liu,* Yan-Ting Lim,‖ Paul MacAry,‖§ and Yunn-Hwen Gan*†§

*Burkholderia pseudomallei* is a Gram-negative saprophyte that is the causative agent of melioidosis, a severe infectious disease endemic in Northern Australia and Southeast Asia. This organism has sparked much scientific interest in the West because of its classification as a potential bioterrorism agent by the U.S. Centers for Disease Control and Prevention. However, relatively little is known about its pathogenesis. We demonstrate that *B. pseudomallei* actively inhibits NF-κB and type I IFN pathway activation, thereby downregulating host inflammatory responses. We found the virulence factor TssM to be responsible for this activity. TssM interferes with the ubiquitination of critical signaling intermediates, including TNFR-associated factor-3, TNFR-associated factor-6, and IκBα. The expression but not secretion of TssM is regulated by the type III secretion system. We demonstrate that TssM is important for *B. pseudomallei* infection in vivo as inflammation in the tssM mutant-infected mice is more severe and corresponds to a more rapid death compared with wild-type bacteria-infected mice. Abs to TssM can be detected in the sera of melioidosis patients, indicating that TssM is functionally expressed in vivo and thus could contribute to bacterial pathogenesis in human melioidosis. *The Journal of Immunology*, 2010, 184: 5160–5171.

The innate immune response represents the first line of defense during infection and uses a number of pathogen-associated molecular pattern receptors that elicit proinflammatory signaling pathways that converge in the activation of NF-κB. NF-κB is a central transcription factor responsible for controlling the expression of multiple genes involved in inflammatory response, such as TNF-α, IL-1, IL-6, IL-8, and IL-12 (1). The importance of NF-κB in resistance to infection is best illustrated in knockout mice deficient in different components of the NF-κB pathway that are susceptible to a variety of infections (2). Because NF-κB plays a critical role in the clearance of the bacteria by the immune response, it is perhaps not surprising that many pathogenic bacteria have evolved mechanisms to manipulate this pathway and dampen the host immune response. Two well-documented bacterial strategies involve secreting effectors with inhibitory Toll/IL-1R homologous domains, such as early secreted Ag target-6 from *Mycobacteria* (3) and TcpS from uropathogenic *Escherichia coli* and *Brucella* (4, 5) and producing effectors that interfere with host ubiquitin (Ub) signaling pathways (6). Bacteria using the latter strategy use the type III secretion systems or type IV secretion systems to inject their effectors (7–9). More recently, SseL of *Salmonella* and ChlDub1 of *Chlamydia* have been shown to inhibit NF-κB through deubiquitination, rendering IκBα insensitive to proteasome degradation (10, 11).

*Burkholderia pseudomallei* is the causative agent of melioidosis, a severe emerging infectious disease that is endemic in Southeast Asia and Northern Australia (12). The case fatality rate of severe melioidosis patients is ~50% in Thailand, and the mortality rate of all patients in Australia approaches 20% (13, 14). The clinical manifestations of melioidosis are extremely varied, ranging from asymptomatic seroconversion, acute and chronic infection, and localized infection involving one organ to disseminated septicemia or septic shock (15). In addition, latency of as long as 24–29 y before disease manifestation in ex-servicemen who were in Papua New Guinea and Vietnam have been described previously (16–18). The factors and mechanisms contributing to these diverse disease outcomes are unknown. *B. pseudomallei* is also able to infect and cause disease in a wide variety of animal species ranging from captive marine animals, dogs, cats, and ruminants to primates (19–23). Because its classification as a potential bioterrorism agent by the U.S. Centers for Disease Control and Prevention (www.cdc.gov) and the completion of its huge 7-Mb genome sequence (24), there has been much scientific interest in understanding the pathogenesis of this versatile bacterium because of its medical importance and its use as a model organism for the study of bacterial pathogenesis. *B. pseudomallei* possesses many virulence factors including three type III secretion systems (25). One of these loci designated the *Burkholderia* secretion apparatus (bsa) gene cluster shares homology with the *Salmonella* typhimurium inv/spa/prg T3SS and is required for cellular invasion and escape from endocytic vesicles (26). This bacterium also possesses six type VI secretion system (T6SS) clusters, and the T6SS-5 has been reported.
to be important for the invasion of macrophages (27). Several lines of experimental evidence suggest that \textit{B. pseudomallei} is able to modulate the innate immune response. \textit{B. pseudomallei} has been shown to induce less IL-8 and iNOS degradation in A549 lung epithelial cells when compared with \textit{Salmonella} (28). Similarly, it induces less inducible NO synthase and TNF-\alpha in RAW264.7 macrophages when compared with other Gram-negative bacteria (29). The muted response was postulated to be because of a less stimulatory nature of \textit{B. pseudomallei} LPS (30).

Our previous work has demonstrated that less NF-\kappaB and IL-8 is induced by \textit{B. pseudomallei} compared with \textit{E. coli} and \textit{Salmonella} in HEK293T cells, which do not express TLR-4, the receptor for LPS (31). In this study, we show that \textit{B. pseudomallei} has the ability to actively attenuate NF-\kappaB and IFN-sensitive response element (ISRE) signaling pathways. We identify the BPSS1512 gene product of \textit{B. pseudomallei} to be responsible for this activity. BPSS1512 is 100% identical to \textit{tssM} of \textit{Burkholderia mallei}, which has recently been shown to encode a broad-base deubiquitinase, which cleave both K48- and K63-linked ubiquitinated substrates during in vitro enzymatic assays, although the authors could not find any cellular substrates or phenotype either in cellular assays or animal models (32, 33). We further demonstrate the importance of \textit{B. pseudomallei} TssM in an acute mouse infection model and establish its functional relevance in human infection based on the detection of anti-TssM Abs in sera of melioidosis patients.

Materials and Methods

Cell culture

RAW 264.7 and HEK 293T cell lines were obtained from the American Type Culture Collection (Manassas, VA). The cells were cultured in DMEM supplemented with 10% heat-inactivated FBS (Life Technologies, Rockville, MD), 2 mM L-glutamine (Life Technologies), and 1× penicillin/streptomycin (Life Technologies) under a humidified atmosphere with 5% CO\textsubscript{2} at 37°C.

Reagents

Pam\textsubscript{3}CSK\textsubscript{4} (PAM), ultrapure LPS from \textit{E. coli} K12, and polyinosine-polycytidylic acid [poly(I:C)] were purchased from InvivoGen (San Diego, CA). Recombinant \textit{B. pseudomallei} flagellin was prepared as described previously (31).

Bacterial strains and mutants

Bacterial strains used in the current study are described in Table 1. \textit{B. pseudomallei} \textit{tssM}, \textit{boa TSS3}, and \textit{TSS5-5} mutants were generated in \textit{KHW} strain (34) by replacement of the respective gene or gene clusters with antibiotic-resistant cassettes. Briefly, ~1-kb fragments upstream and downstream of the genes were amplified from genomic DNA and cloned into pK18mobsacB (35). The tet cassette from pFRT T1 or zeocin cassette (zoe) (a gift from Prof. H. Schweizer, Colorado State University, Fort Collins, CO) from pCLOXZ1 was inserted between the gene fragments. The plasmids were electroporated into \textit{B. pseudomallei} strain \textit{KHW}. Homologous recombination was selected for retention of \textit{tet} or \textit{zoe} markers and loss of the plasmid marker (Km) to generate KHW\textit{tssM::tet}, KHW\textit{TSS3::zoe}, and KHW\textit{TSS5::tet}. Deletion of the chromosomally integrated \textit{tet} marker in KHW\textit{TSS5::tet} and KHW\textit{TSS3::tet} by Flp recombinase-catalyzed excision was achieved by conjugally transferring the Flp-expressing pLP2km from SM10 into the \textit{tet}-resistant mutants. The excision was screened for loss of antibiotic marker (\textit{tet}) linked to the mutation and loss of the plasmid marker (Km) to generate KHW\textit{tssM} and KHW\textit{TSS5}. The identity of the mutants was confirmed by PCR for the loss of target genes.

Cloning, plasmids, and transfection

All plasmids used in this study are described in Table 1. Flag- TNFR-associated factor (TRAF-6) and hemagglutinin (HA)-Ub plasmids were generously provided by Profs. P. Cohen (University of Dundee, Dundee, Scotland) and C. N. Ong (National University of Singapore [NUS], Singapore), respectively (36). Flag-IksB\alpha plasmid was obtained from subcloning of IksB\alpha plasmid (IMAGE 2957970) into \textit{HindIII} and XbaI restriction enzyme sites of pFlag-CMV expression vector (Sigma-Aldrich, St. Louis, MO).

Flag-TRAF-3 plasmid was generated from subcloning of TRAF-3 plasmid (IMAGE 30915387) into \textit{HindIII} and BunHI restriction enzyme sites of pFlag-CMV expression vector. BPSS152 was cloned into mammalian expression vector pCMV-TOPO (Invitrogen, Carlsbad, CA) by TA cloning. For overexpression of \textit{TssM} in \textit{B. pseudomallei}, \textit{tssM} was cloned into pMLBAD vector (a gift from Prof. M. A. Valvano, University of Western Ontario, London, Ontario, Canada) (37). HEK293T and RAW 264.7 cells transfection were carried out using FuGene HD (Roche, Basel, Switzerland). Secreted alkaline phosphatase (SEAP) activity in the culture supernatant was measured using a Phospha-Light assay kit (Applied Biosystems, Foster City, CA), according to the manufacturer’s protocol.

Stable cell line

RAW 264.7 cells stably expressing NF\textit{\kappaB-SEAP} reporter (BD Clontech, Palo Alto, CA) was generated by transfecting cells with the NF\textit{\kappaB-SEAP} reporter plasmid containing a neomycin cassette, which confers resistance to geneticin. NF\textit{\kappaB-SEAP} with a neomycin cassette was obtained through subcloning of neomycin cassette from pGeneClip neomycin (Promega, Madison, WI) into the Sall restriction enzyme site of NF\textit{\kappaB-SEAP} plasmid. Stable cell line was routinely maintained in DMEM complete supplemented with 500 \mu g/ml geneticin (Invitrogen).

ELISA

NF-\kappaB and IFN-\beta proteins were determined in the culture supernatant of RAW cells by ELISA using kits from BenderMed Systems (Vienna, Austria) and PBL Interferon Source (Piscataway, NJ), respectively, according to the manufacturer’s instructions in triplicate.

Bacterial infection

Cells were infected with log-phase culture of \textit{B. pseudomallei} at the required multiplicity of infection (MOI) in antibiotics-free medium. Midlog-phase bacteria were prepared by inoculating 250 \mu l overnight culture into 5 ml Luria-Bertani (LB) broth and allowed to grow for 2 h with constant agitation. Infected cells were centrifuged at 200 \times g for 5 min to allow bacteria to cell contact. One hour following infection, 250 \mu g/ml kanamycin was added to kill off extracellular bacteria.

Immunoblotting and immunoprecipitation

For immunoprecipitation, transfected cells were lysed in immunoprecipitation buffer (Sigma-Aldrich) supplemented with protease inhibitor mixture. Cell lysates were incubated with anti-Flag agarose (Sigma-Aldrich) for 3 h at 4°C. The identity of the mutants was confirmed by immunoblotting as described above. Flag-TRAF-3 plasmid was generated from subcloning of TRAF-3 plasmid (IMAGE 30915387) into \textit{HindIII} and BunHI restriction enzyme sites of pFlag-CMV expression vector. BPSS152 was cloned into mammalian expression vector pCMV-TOPO (Invitrogen, Carlsbad, CA) by TA cloning. For overexpression of \textit{TssM} in \textit{B. pseudomallei}, \textit{tssM} was cloned into pMLBAD vector (a gift from Prof. M. A. Valvano, University of Western Ontario, London, Ontario, Canada) (37). HEK293T and RAW 264.7 cells transfection were carried out using FuGene HD (Roche, Basel, Switzerland). Secreted alkaline phosphatase (SEAP) activity in the culture supernatant was measured using a Phospha-Light assay kit (Applied Biosystems, Foster City, CA), according to the manufacturer’s protocol.

Expression and purification of rtTssM protein

The \textit{tssM} gene was cloned in frame with N terminus His-tagged of pET-28a expression plasmid and transformed into BL21 (DE3) for protein expression and purification. Purification of rtTssM was achieved using His-tagged purification system (Pierce Chemical, Rockford, IL).

Production of polyclonal Abs against TssM

Six female BALB/c mice were injected i.p. with 25 \mu g rtTssM each in CFA (Sigma-Aldrich). After 3 wk, the mice were boosted once i.p. with 25 \mu g rtTssM in IFA (Sigma-Aldrich), followed by a second boost 2 wk thereafter. A final boost with 25 \mu g rtTssM in PBS was injected i.v. 1 wk after the second boost. Cardiac puncture was performed on the mice, and serum samples were pooled.

Detection of TssM secretion

Overnight wild-type and mutant \textit{B. pseudomallei} strains were diluted 1/20 and cultured for 3 h in LB broth. Culture supernatants were filter sterilized by passing through 0.22-\mu m Millipore filters, and proteins were precipitated using 10% trichloroacetic acid. Protein pellets were collected by centrifugation and washed twice with ice-cold acetone before being air-dried and resuspended in SDS-PAGE sample buffer and analyzed by immunoblotting as described above.
RNA isolation and quantitative real-time PCR

Total RNA was isolated using Micro to Midi RNA extraction kit (Invitrogen). Prior to reverse transcription, RNA was treated with 2 U TURBO DNase I (Ambion, Austin, TX) at 37˚C for 30 min. Reverse transcription was performed using High Capacity cDNA synthesis kit (Applied Biosystems). Transcripts were quantified by real-time PCR using SYBRgreen (Invitrogen) in a BioRad iQ5 machine (Bio-Rad, Hercules, CA). The expression mRNAs were normalized to the relative abundance of the relevant housekeeping gene. Fold induction was calculated using the $2^{-ΔΔCt}$ method (38).

Animal studies

Female 6- to 8-wk-old BALB/c mice were purchased from the Center for Animal Resources of NUS. The mice were fed with irradiated commercial pellets (PMI Nutrition International, St. Louis, MO) and potable water ad libitum. All experimental procedures with mice and infection were approved by the Animal Care and Use Committee, Defence Science Organization National Laboratories and the NUS Institutional Animal Care and Use Committee. Mice were lightly anesthetized with 3% isoflurane in oxygen before being inoculated with 100 CFU bacteria through one nostril of each mouse with a pipette tip in 10 µl PBS.

Organ loads

Lungs and spleens were removed from the mice 48 h postinfection and homogenized in 1× PBS. Serial dilution was performed, followed by plating on Ashdown plates. Data shown are combination of two experiments. Missing data points are due to errors in dilutions and plating or difficulty encountered during isolation of organs.

Histopathology

Spleens and lungs were harvested from wild-type and tssM mutant-infected mice at two time points, 48 h postinfection or at the time of death of animals. Organs were fixed in 10% buffered formalin and embedded in paraffin. Paraffinized sections of 3-µm thickness were deparaffinized, rehydrated through graded alcohol to water, and stained with H&E. The lungs and spleen sections were analyzed and scored for three parameters: intensity of interstitial and perivascular inflammatory infiltrate, formation of abscesses, exudation in airways (for lung sections only), and thrombus formation.
formation (for splenic sections only). Each parameter is graded on a scale of 0–3, and their criteria were defined as follows: 1) inflammation: 0 = no inflammation; 1 = some inflammatory infiltrates; 2 = moderate number of inflammatory infiltrates; and 3 = high number of inflammatory infiltrates; 2) abscess: 0 = no abscess; 1 = 1 or 2 small abscesses; 2 = 1–3 abscesses of moderate size; and 3 = 3 abscesses/multiple foci or presence of very large abscesses; 3) bronchial inflammation: 0 = no inflammatory infiltrate in airways; 1 = presence of inflammatory cells within 1 area of airway; 2 = presence of inflammatory cells in 2 or more foci; and 3 = high number of inflammatory cells within airways; and 4) thrombosis: 0 = no thrombus identified; 1 = small blood clots in one area; 2 = small clots in more than one area; and 3 = presence of clots in larger vessels. The maximum total histology score for each organ is 9.

Detection of anti-TssM Ab in patient sera

All five patients had diabetes mellitus or renal disease and were culture positive for \textit{B. pseudomallei}, with an immunohemagglutination assay (IHA) titer ranging from 1/128 to 1/1024. IHA measures Abs to whole-killed bacteria. Sera were collected from patients 2 wk after hospital admission and after antibiotic administration. Sera from five healthy volunteers were included as negative controls. \textit{rTssM} was subjected to immunoblotting as described above. Membranes were probed with 1/1,000 dilutions of patient sera for 1 h at room temperature and with a 1/10,000 dilution of HRP-labeled sheep anti-human IgG (GE Healthcare) and developed as described above.

Statistical analysis

Statistical significance was determined by performing Student $t$ test. Differences were considered significant if $p < 0.05$.

Results

\textit{B. pseudomallei} suppresses NF-κB activation

\textit{B. pseudomallei} was shown to be less stimulatory to epithelial cells and macrophages compared with other Gram-negative bacteria (28, 29). We have previously reported that \textit{B. pseudomallei} activates low levels of NF-κB via TLR2, TLR4, and TLR5 (31). To determine whether the muted activation in cells is due to an active suppression exerted by the bacteria, we stimulated HEK293T cells transfected with the relevant TLRs with their respective TLR agonists and infected the cells with \textit{B. pseudomallei}. \textit{B. pseudomallei} suppressed TLR2-, TLR4-, and TLR5-mediated NF-κB activation potently at an MOI of 10:1 (Fig. 1, A–C). We next investigated the activity of NF-κB in RAW264.7 macrophages infected with \textit{B. pseudomallei}, because these cells express TLRs naturally. RAW cells infected with KHW exhibited a 2-fold increase in NF-κB activity, whereas

![FIGURE 2. TssM inhibits TLR2-, TLR4-, TLR5- and TNF-α-mediated NF-κB activation by deubiquitination of TRAF-6 and IκBa. RAW cells (A) and HEK293T cells (B) were transiently cotransfected with NFκB-SEAP reporter and \textit{tssM}-expressing plasmids. Twenty-four hours posttransfection, cells were treated with TLR agonists or TNF-α for 6 h before culture supernatants were subjected to SEAP reporter assay. Experiments were carried out in triplicate and averaged, then converted to fold activation with respect to the control. HEK293T cells were transiently cotransfected with plasmids expressing \textit{TssM}, HA-Ub, and Flag-TRAF-6 (C) or Flag-IκBa (D). Concentration of \textit{tssM} plasmid used was 125 ng unless otherwise indicated. Twenty-four hours after transfection, cells were lysed, and lysates were subjected to immunoprecipitation using anti-Flag Ab, after which membrane was stripped and reprobed with anti-HA Ab. All results shown are representative of three independent experiments.](http://www.jimmunol.org/)

![FIGURE 3. Overexpression of TssM inhibits ISRE signaling and TRAF-3 ubiquitination. A. RAW cells were transiently transfected with ISRE-SEAP reporter 24 h prior to infection. Cells were infected with bacteria at the indicated MOI for 6 h. Experiments were carried out in triplicate and averaged, then converted to fold activation with respect to poly(I:C) treatment alone. B. HEK293T cells were transiently transfected with plasmids expressing \textit{tssM}, HA-Ub, and Flag-TRAF-3. Twenty-four hours after transfection, cells were lysed, and lysates were subjected to immunoprecipitation using anti-Flag Ab, after which membrane was stripped and reprobed with anti-HA Ab. The results shown are representative of three independent experiments.](http://www.jimmunol.org/)
heat-killed (HK) *B. pseudomallei* activated NF-κB by 4.5-fold (Fig. 1D). Both *E. coli* and *Salmonella enterica* activated NF-κB by 6-fold (Fig. 1D). Furthermore, *B. pseudomallei* inhibited PAM, ultrapure LPS, and flagellin-mediated NF-κB activation at an MOI of 100:1 and even at a lower MOI of 10:1 (Fig. 1E). Consistent with the results obtained from the reporter assay, live *B. pseudomallei* inhibited LPS-mediated phosphorylation of IKKα/β and IκBα whereas HK bacteria could not (Fig. 1F). Bacteria induced <1% cytotoxicity in RAW cells at MOIs of 10:1 and 100:1 at these and all subsequent infection experiments (data not shown), excluding the possibility that NF-κB inhibition in macrophages is attributed to cell death. No cell death was observed for infected HEK293T cells for all our experiments. Because inhibition was obvious with an MOI of 10:1, all subsequent experiments were carried out using this lower ratio of bacteria to host cells.

*TssM inhibits NF-κB activation via TLR and TNF-αR by interfering with TRAF-6 and IκBα ubiquitination*

Bacterial pathogens have been documented to suppress NF-κB signaling pathway by interfering with ubiquitination (6) and phosphorylation (39) of signaling intermediates and by disrupting signaling complexes through homotypic interaction with Toll/IL-1R domains of signaling molecules (4, 40). To identify the effector responsible for NF-κB suppression in *B. pseudomallei*, we performed homology searches for similar proteins in *B. pseudomallei* and transiently overexpressed putative effectors in RAW264.7 cells. We identified one effector that inhibited PAM-, LPS-, and flagellin-mediated NF-κB activation in RAW264.7 cells stably transfected with an NFκB-dependent luciferase reporter (Fig. 2A). The gene (BPSS1512) encoding this effector is 100% identical to the previously described *B. mallei tssM* gene (33). A sequence analysis revealed that the tssM of KHW strain is identical to that of genome reference strain K96243. When overexpressed in HEK293T cells, *tssM* also potently inhibited TNF-α–mediated NF-κB activation (41). The K48-linked polyubiquitination of IκBα is necessary for proteasome degradation, whereas the K63-linked polyubiquitination of TRAF-
6 is required for IKK catalytic activation (42). To test whether ubiquitinated TRAF-6 is a substrate for TssM, Flag-TRAF-6 was overexpressed with HA-Ub and TssN in HEK293T cells, and ubiquitinated TRAF-6 was analyzed by immunoprecipitation using an anti-Flag Ab and immunoblotting with anti-HA Ab. Ubiquitinated forms of TRAF-6 were observed in the absence of TssM and were clearly diminished in the presence of increasing plasmid concentrations of TssM (Fig. 2C). To determine whether IκBα is also a substrate of TssM, Flag-IκBα was overexpressed with HA-Ub and TssM, and ubiquitinated IκBα was assayed following TNF-α treatment to induce IκBα phosphorylation and ubiquitination. Immunoblotting of immunoprecipitated Flag-IκBα shows that overexpression of TssM resulted in significantly less ubiquitinated IκBα (Fig. 2D).

**TssM inhibits ISRE signaling**

TLR3 and TLR4 stimulation activates IFN regulatory factor (IRF)-3 or IRF-7, leading to the transcription of genes possessing an ISRE. Transient overexpression of TssM in RAW cells resulted in the inhibition of TLR3-mediated ISRE activation via poly(I:C) (Fig. 3A). Activation of ISRE through TLR3 and TLR4 occurs through a common signaling intermediate TRAF-3, which is modified by ubiquitination (43). To determine whether TssM is capable of deubiquitinating TRAF-3, HEK293T cells were transfected with Flag-TRAF-3, HA-Ub, and TssM. Like TRAF-6, ubiquitinated forms of TRAF-3 were also diminished in the presence of TssM, suggesting that TssM can deubiquitinate the K63-linked polyubiquitinated chain on TRAF-3 (Fig. 3B).

**Live bacterial suppression of NF-κB and ISRE is mediated by TssM**

To evaluate the contribution of TssM to NF-κB suppression during a live bacterial infection, a tssM null mutant was created in a wild-type bacteria background by replacing the tssM gene with a tet gene cassette and subsequently flipping this out to create an unmarked mutant (Table I) (44). RAW cells were infected with wild-type bacteria or tssM mutant and assayed for NF-κB and ISRE reporter activities. We found that tssM mutant was able to relieve the NF-κB suppression seen in wild-type bacteria when stimulated with PAM, LPS, and flagellin (Fig. 4A). tssM mutant was also able to relieve the ISRE suppression seen in wild-type bacteria when stimulated with a TLR3 agonist (Fig. 4B). Deletion of tssM gene
in *B. pseudomallei* does not impair intracellular replication of the bacteria in macrophages (Fig. 4C). Restoration of *tssM* gene function by overexpressing TssM in the *tssM* mutant restored the ability of the mutant to inhibit NF-κB and ISRE reporter activities (Fig. 4A, 4B). Furthermore, RAW cells infected with the *tssM* mutant produced more TNF-α and IFN-β compared with cells infected with wild-type bacteria (Fig. 4D, 4E). These results strongly suggest that TssM is critical for NF-κB and ISRE suppression in *B. pseudomallei*.

**Regulation and secretion of TssM**

Because *tssM* is located immediately downstream of the T6SS-5 locus and before the *bsa* T3SS cluster (32), we determined whether its regulation is affected by either of these two clusters. To create mutants lacking the entire reported *bsa* T3SS and T6SS-5 clusters, we select for homologous recombination between the targeting plasmid carrying the upstream and downstream flanking fragments with the antibiotic resistance cassette and genomic DNA, as shown in the schematic figures (Fig. 5A, 5B). We found that *tssM* expression in wild-type bacteria was induced 9-fold following infection of RAW cells (Fig. 5C). *tssM* expression was unchanged in the T6SS-5 mutant but reduced by ~10-fold in the *bsa* T3SS mutant compared with the wild-type bacteria (Fig. 5C). The reduction in expression in the T3SS mutant was not due to decreased bacterial load in macrophages because T3SS mutant replicated to similar levels as wild-type bacteria at 3 h post-infection (data not shown). Thus, *tssM* is transcriptionally regulated by the *bsa* T3SS but not the T6SS-5 locus. For TssM to function as a deubiquitinase on host signaling molecules, it would have to be present in the cytosol of infected cells. Hence, we determine whether TssM is a secreted effector by testing for its presence in the supernatant of wild-type bacteria, the *bsa* T3SS,

**FIGURE 5.** *tssM* gene expression is regulated at the transcriptional level by *bsa* T3SS, but protein secretion is *bsa* T3SS- and T6SS-5 independent. Schematic diagrams (not drawn to scale) showing the generation of *bsa* T3SS (A) and T6SS-5 mutants (B). Small vertical lines represent primer sites for generation of upstream and downstream fragments. Dotted arrow in B indicates the additional step of flipping out the tet cassette flanked by FRT sites. C, RAW cells were infected with KHW, T3SS, or T6SS mutants at MOI 10:1 for 3 h. Cells were washed extensively with 1× PBS to remove extracellular bacteria prior to cell lysis and RNA extraction. D, KHW and *ΔtssM* were subcultured in LB broth for 3 h before cells were lysed, and lysates were subjected to Western blot analysis using anti-TssM Ab. E, Culture supernatants from wild-type bacteria KHW, *ΔT3SS*, *ΔT6SS*, and *ΔtssM* mutants were precipitated and subjected to Western blot analysis using anti-TssM Ab. Blot shown is representative of three independent experiments.
and T6SS-5 mutants growing in LB medium. As expected, we found that TssM is present in the bacterial lysate of the wild-type bacteria but not the tssM mutant (Fig. 5D). Interestingly, secretion of TssM is independent of bsa T3SS-3 and T6SS-5 (Fig. 5E). The presence of three bands for TssM in B. pseudomallei was also seen for TssM in B. mallei, likely representing processed derivatives of the secreted protein (33).

**Role of TssM during in vivo mouse infection**

To determine the role of TssM during an in vivo infection, we infected BALB/c mice with either wild-type bacteria or the tssM mutant. Significantly higher levels of TNF-α and IL-6 mRNA were observed in the lungs of those infected with the mutant versus those infected with the wild-type bacteria (Fig. 6A, 6B). In addition, higher expression of IFN-β and IL-6 transcripts were also observed in the spleen of tssM mutant-infected mice compared with wild-type bacteria-infected mice (Fig. 6C, 6D). There were no differences in expression of IFN-γ, IL-10, IL-12p35, KC, and Rantes between mutant and wild-type–infected mice (data not shown). Mice infected with tssM mutant died earlier than mice infected with wild-type bacteria (Fig. 6E). The median survival for tssM mutant and wild-type–infected mice were 4 and >8.5 d, respectively, representing a statistically significant difference. The increased expression of selective cytokines in the lungs and spleens of tssM mutant-infected mice is unlikely to be due to higher bacterial growth because the bacterial loads in the lungs and spleens of wild-type versus mutant infected mice were not statistically significant at 48 h postinfection (Fig. 6F).

Histological analysis of lungs and spleens of mice infected with wild-type or tssM mutant bacteria revealed extensive inflammatory changes (Fig. 7). However, tssM mutant-infected mice showed significantly more inflammation in the spleens (but not the lungs) compared with wild-type–infected mice at both 48 h postinfection and at the point of death (Fig. 7A, 7B). Inflammatory infiltrate was not seen in the alveolar spaces in any of the lung sections examined from both groups of mice (data not shown). Thrombosis was detected in the spleens of tssM mutant-infected mice at the point of death but not at 48 h postinfection (Fig. 7F). Taken together, our results show that the loss of TssM correlated with hyperinflammation of the host in vivo and hyperinflammation correlated with a faster death.

**TssM is expressed during in vivo human infection**

To determine the relevance of TssM to human infection, we tested for anti-TssM Abs in the sera of culture-positive melioidosis patients who presented with acute or subacute disease. Three of five patients had anti-TssM Abs when they were tested 2 wk after admission into hospitals (Fig. 8), and none of the five healthy controls had the Abs. This shows that TssM is expressed and likely secreted during human infection.

**Discussion**

Modification of cellular proteins by ubiquitination is a process restricted to eukaryotes and does not occur in prokaryotes.
Ubiquitination involves the conjugation of Ub moieties onto a target protein. Polyubiquitin chains linked through their lysine 48 (K48) are targeted for proteasome degradation, whereas K63-linked chains are known to activate and modify protein activity (45). In mammalian cells, ubiquitination involves three enzymatic steps. Ub-activating enzyme (E1) transfers Ub to E2-conjugating enzymes, which then transfer the activated Ub to a lysine residue of the target protein through E3 ligases. Bacteria have evolved strategies to modulate host signaling pathway through virulence factors with deubiquitinase activity (6). In this study, we describe how \( B. \ pseudomallei \) modulates the host innate immune response through the bacterial protein TssM. TssM alone impaired proinflammatory cytokine and IFN-\( \beta \) secretion during infection of macrophages. Overexpression of TssM leads to the deubiquitination of TRAF-6, TRAF-3, and I\( \kappa \)B and thereby interferes with the activation of NF-\( \kappa \)B and ISRE pathways.

We found that \( B. \ pseudomallei \)-infected macrophages produced undetectable amounts of IFN-\( \beta \). Recent evidence suggests that type I IFNs are involved in the response to bacterial as well as viral infections. In fact, IFN-\( \beta \) has been reported to possess potent antimicrobial properties in infected macrophages (47). IFN-\( \beta \) shows protective effects against \( Listeria \) monocytocegenes and \( Toxoplasma \) gondii in infected mice (48, 49). Mice infused with IFN-\( \beta \) showed reduced growth of \( Mycobacterium \) avium in spleens and livers (50). IFN-\( \beta \) promoter contains NF-\( \kappa \)B binding sites, a site for AP-1 and two ISREs recognized by phosphorylated IRF-3 (51).

**FIGURE 7.** Histology of organs from wild-type and \( tssM \) mutant infected mice were carried out using H&E staining. Original magnifications \( \times60 \) (C, D) and \( \times20 \) (E, F). Lungs and spleens from BALB/c mice were harvested either 48 h postinfection or at the point of death of the animal. Histology scores of infected lungs (A) and spleen (B) were calculated as described in Materials and Methods. Representative lung and spleen histology of wild-type (C, E) and \( tssM \) mutant (D, F) infected mice are shown. Abscesses (A) were seen in the lungs and spleens of mice infected with wild-type bacteria and \( tssM \) mutant. Areas of acute inflammation and vascular congestion were seen in all the lung samples. The inflammatory infiltrates consisted mainly of neutrophils and monocytoid cells (C, D). Exudate (E) was detected in the bronchial lumen in some wild-type and mutant infected animals. F, Thrombus (T) was detected in two of the four \( tssM \) mutant-infected spleens examined. R, red pulp; S, alveolar space; W, white pulp.

**FIGURE 8.** Reactivity of sera from melioidosis patients to rTssM protein. Abs reactive to rTssM were detected in the sera of patients 1, 3, and 5, whereas sera from patients 2 and 4 were not reactive.
NF-κB and IFN pathways has been described previously (52). Although phosphorylated IRF-3 can bind to the ISRE site on IFN-β promoter, it does not stimulate transcriptional activity. It has been shown that a fusion protein generated from the IRF-3 binding domain and the p65 activation domain activates IFN-β promoter, indicating that IRF-3 needs to cooperate with p65 to stimulate transcription from the IFN-β promoter (53). Therefore, we predict that in addition to the direct deubiquitination of TRAF-3 by B. pseudomallei, inhibition of the NF-κB pathway through deubiquitination of TRAF-6 and IkBα could further dampen the IFN-β response to the bacteria by blocking p65-mediated transactivation of ISRE signaling.

B. pseudomallei activates TLR2, TLR 4, and TLR 5 in vitro (31, 54). In vivo mouse infection showed that the absence of TLR4 did not accelerate disease or mortality, whereas the absence of TLR2 was protective (55). With the exception of TLR3, all other TLRs signal through the MyD88 adaptor, whereas TLR3 and TLR4 signal through the TRIF adaptor. Recruitment of MyD88 and TRIF to the respective TLRs leads to the polyubiquitination of TRAF-6 and TRAF-3, respectively. Recently, it has been shown that MyD88 but not TRIF-dependent signaling contributes to a protective host response against B. pseudomallei in mice (56). Thus, the protection provided by MyD88 signaling must come from TLRs other than TLR2 or TLR4, at least in mice. Because overexpression of TssM leads to deubiquitination of both TRAF-6 and TRAF-3, B. pseudomallei infection is likely to attenuate TRIF signaling and TLR-mediated NF-κB and ISRE activation. Furthermore, secondary activation of NF-κB through cytokines, such as TNF-α and IL-1β, could also be attenuated as TssM also interferes with IkBα ubiquitination and activation.

In fact, this seems to be supported by our animal experiments, which show that in an acute mouse infection model, the absence of TssM increases the rate of death and inflammation. The higher amount of TNF-α, IL-6, and IFN-β cytokines found in tssM−/−infected mice confirms our work in RAW264.7 cells and suggests that the loss of TssM in vivo contributes to more inflammation in an acute B. pseudomallei infection. It is likely that the loss of TssM contributed directly to increased NF-κB and ISRE activity, thus leading to more TNF-α and IL-6 and increased IFN-β, respectively, rather than resulting in more bacterial growth, which in turn led to hyperinflammation. This is supported by our finding that there was no significant differences in bacterial organ loads between tssM−/−infected or wild-type bacteria-infected mice. Furthermore, if the latter case were true, all the cytokines and chemokines examined should be increased in the mutant-infected mice when compared with wild-type–infected mice (34, 57, 58). Hyperinflammation in acute melioidosis corresponded with increased tissue pathology and had been proposed to contribute to host tissue damage and death (34, 57–59). Following acute infection, the loss of TssM could also be correlated with increased tissue pathology as demonstrated in the spleens of tssM−/−infected mice and accelerated host death. Similarly, Salmonella sseL mutant had been shown to cause an increase rate of death, although the difference between the wild-type bacteria and the mutant is not as marked as with B. pseudomallei (10). This could be because SseL exhibits a narrower range of substrate specificity than TssM. The Shigella flexneri ospG mutant has been shown in a rabbit ileal loop model to cause increased inflammation compared with wild-type bacteria as OspG could prevent phospho-IκBα degradation by targeting Ub-conjugating enzymes (60). Accelerating host death is unlikely to be beneficial to the pathogen, and TssM could act as a virulence factor to inhibit overt inflammation, thus allowing more time for perpetuation of the bacterial infection. It remains to be tested whether the role of TssM as a virulence factor would be clearer under chronic me-