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Original article

TLR4 genetic variation is associated with inflammatory responses in Gram-positive sepsis

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ABSTRACT

Objectives: To identify important pathogen recognition receptor (PRR) pathways regulating innate immune responses and outcome in *Staphylococcus aureus* sepsis.

Methods: We analysed whether candidate PRR pathway genetic variants were associated with killed *S. aureus*-induced cytokine responses *ex vivo* and performed follow-up *in vitro* studies. We tested the association of our top-ranked variant with cytokine responses and clinical outcomes in a prospective multicentre cohort of patients with staphylococcal sepsis.

Results: An intronic *TLR4* polymorphism and expression quantitative trait locus, rs1927907, was highly associated with cytokine release induced by stimulation of blood from healthy Thai subjects with *S. aureus ex vivo*. *S. aureus* did not induce *TLR4*-dependent NF- κ B activation in transfected HEK293 cells. In monocytes, tumor necrosis factor (TNF)- α release induced by *S. aureus* was not blunted by a *TLR4*/MD-2 neutralizing antibody, but in a monocyte cell line, TNF- α was reduced by knockdown of *TLR4*. In Thai patients with staphylococcal sepsis, rs1927907 was associated with higher interleukin (IL)-6 and IL-8 levels as well as with respiratory failure. *S. aureus*-induced responses in blood were most highly correlated with responses to Gram-negative stimulants whole blood.

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Conclusions: A genetic variant in *TLR4* is associated with cytokine responses to *S. aureus* *ex vivo* and plasma cytokine levels and respiratory failure in staphylococcal sepsis. While *S. aureus* does not express lipopolysaccharide or activate TLR4 directly, the innate immune response to *S. aureus* does appear to be modulated by *TLR4* and shares significant commonality with that induced by Gram-negative pathogens and lipopolysaccharide. **N. Chantratita, CMI 2017;23:47.e1–47.e10**

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Introduction

The incidence of Gram-positive sepsis has been continually increasing in recent years and is the predominant type of sepsis in the United States [1]. *Staphylococcus aureus* is the most common Gram-positive organism implicated in severe sepsis, with a mortality rate of 30% [2]. Infection with *S. aureus* can be acquired in both community and healthcare settings, and methicillin resistance in both arenas is a major ongoing concern. A better understanding of the host–pathogen interaction that may lead to novel approaches to treating Gram-positive sepsis is therefore of paramount importance.

Pattern recognition receptors (PRRs) on host immune cells mediate much of the inflammatory response to infection upon activation by conserved pathogen-associated molecular patterns (PAMPs) [3]. The PAMP-PRR interaction is a central driver of the host response in sepsis, and a better comprehension of the PAMP-PRR axis in human sepsis may unveil new targets for therapeutic exploitation. As a Gram-positive organism, *S. aureus* cell wall components activate membrane-bound PRRs such as Toll-like-receptor (TLR) 2 (as a heterodimer with TLR1 or TLR6) and cytoplasmic NOD-like receptors [4]. Many of these PRRs are also activated by PAMPs of Gram-negative organisms [3]. Lipopolysaccharide (LPS) has long been considered to be an essential precipitant of the host inflammatory response to Gram-negative infection, however [5]. In contrast to Gram-negative organisms, *S. aureus* does not express LPS. Although various PRRs may be activated by different bacteria, downstream signaling pathways may converge or be interrelated [3]. It remains unclear whether and how the fundamental inflammatory pathways activated in Gram-positive and Gram-negative sepsis differ from one another and drive outcome [6].

Innate immune genetic variation modulates the host response to infection [7]. We hypothesized that key PRR genes regulating inflammatory responses to *S. aureus* sepsis could be identified by identifying human genetic variation associated with cytokine production and clinical outcome. To test this hypothesis, we assessed the relationship between *ex vivo* blood cytokine responses to *S. aureus* and PRR pathway genetic variation in a large cohort of healthy individuals, then validated our findings in a prospective multicentre cohort of patients with staphylococcal sepsis.

Methods

Ex vivo blood stimulation assays

Three hundred healthy Thai subjects donating blood at the blood donation centre at Sunpasitthiprasong Hospital, Ubon Ratchathani, Thailand, were recruited for a blood sample as previously reported [8]. Those who met enrollment criteria gave written informed consent to participate and provided a postdonation blood sample in citrate tubes. A batch of 96-well immunoassay plates was generated by adding 20 μ L of stimuli in appropriate concentrations to each

well. Plates were frozen at -80°C until the day of use, when they were thawed to 37°C . Fresh whole blood (380 μ L) anticoagulated with citrate and mixed 1:1 with RPMI media was added to each well [8]. Final concentrations of stimuli were as follows: whole heat-killed *S. aureus* strain Newman 2.5×10^7 CFU/mL and 5.0×10^7 CFU/mL, whole heat-killed *Burkholderia pseudomallei* K96243 2.5×10^7 CFU/mL, *Salmonella typhimurium* flagellin 500 ng/mL, Pam3CSK4 100 ng/mL (InvivoGen, San Diego, CA, USA), Pam2CSK4 100 ng/mL (InvivoGen), MDP (10-muramyl dipeptide) 10 μ g/mL (InvivoGen), TriDAP 10 μ g/mL (InvivoGen), *Escherichia coli* O111:B4 LPS 10 ng/mL (List Biological Laboratories, Campbell, CA, USA) and *Salmonella minnesota* Re595 LPS 10 ng/mL (List Biological Laboratories). Plates were placed on a shaker at 37°C under 5% CO_2 for 6 hours before being spun down; the plasma supernatant was removed and frozen at -80°C . Monocyte counts were performed in the hospital clinical laboratory.

Staphylococcal sepsis study

A prospective observational study of community onset *S. aureus* sepsis was conducted at four hospitals in northeast Thailand between March 2010 and December 2013 [9]. Study sites were Sunpasitthiprasong Hospital, Ubon Ratchathani; Udon Thani Hospital, Udon Thani; Srinagarind Hospital, Khon Kaen; and Khon Kaen Hospital, Khon Kaen. Potential study patients were identified by daily screening at each hospital diagnostic microbiology laboratory for clinical samples taken from sterile sites that grew a pure growth of *S. aureus*. Subsequent genetic analysis of isolates revealed that a minority subset (19%) were *S. argenteus* [9], a recently described closely related *Staphylococcus* species that is indistinguishable from *S. aureus* in the diagnostic laboratory. Written informed consent was obtained from all patients or their designee. Inclusion criteria were as follows: age at least 14 years (who are admitted to the adult wards), positive culture taken within 2 days of hospital admission or after 2 days when sampling from a patient admitted with suspected infection was delayed and at least two of four systemic inflammatory response criteria met within 48 hours of culture. These criteria were as follows: temperature $<36^{\circ}\text{C}$ or $>38^{\circ}\text{C}$, heart rate >90 beats per minute, respiratory rate >20 breaths per minute or PaCO_2 <32 mm Hg or requiring mechanical ventilation, white blood cell count <4000 or $>12\,000$ cells/mL or $>10\%$ band forms. Baseline clinical information was obtained from the medical records. Blood was drawn at the time of enrollment, and plasma was separated and frozen at -80°C . Patients were followed until hospital discharge, and mortality was ascertained on the 28th day. Respiratory failure was defined as requirement for mechanical ventilation; shock was defined as requirement for vasopressors or inotropes.

LPS and TLR4-stimulation assays

The presence of LPS in the heat-killed *S. aureus* strain Newman preparation was determined by *Limulus* Amebocyte Lysate gel-clot

assay (Associates of Cape Cod, East Falmouth, MA, USA) according to the manufacturer's instructions (sensitivity 0.03 EU/mL). TLR4-dependent NF- κ B activation by *S. aureus* was assessed by stimulation of hTLR4/hMD-2/hCD14-transfected HEK293 cells, using a modified version of a previous approach [10]. Cells were cultured in a 96-well flat-bottomed tissue culture plate at 35 000 cells per well in Dulbecco modified Eagle medium (DMEM) plus 10% fetal bovine serum and 1% L-glutamine. The following day, cells were transiently transfected with 100 μ L of transfection mixture comprising plasmids, transfection reagent (Polyfect; Qiagen, Valencia, CA, USA) and DMEM. DNA was added in the following amounts per well: ELAM (firefly luciferase) (50 ng), pRL-TK (*Renilla* luciferase) (5 ng), hTLR4 or empty vector (10 ng), hCD14 (1 ng) and hMD2 (1 ng). After 48 hours of incubation, transfected cells were stimulated with heat-killed *S. aureus* or *E. coli* O111:B4 LPS (List Biological Laboratories, Campbell, CA, USA) as a positive control. After 4 hours, cells were washed with phosphate-buffered saline and lysed with passive lysis buffer (Promega, Madison, WI, USA), and NF- κ B activation was determined in 10 μ L of lysate by the ratio of firefly to *Renilla* luciferase light emission by the use of the Dual-Luciferase reporter system (Promega).

Primary monocyte/monocyte cell line stimulations and TLR4 knockdown

Peripheral blood mononuclear cells were isolated from blood obtained from fasting subjects who avoided recent strenuous exercise drawn into 8 mL Vacutainer CPT tubes (Becton Dickinson (BD), San Diego, CA, USA). Subjects were between 18 and 65 years of age, weighed between 100 and 350 pounds, were of Southeast Asian ancestry, had no chronic medical conditions, were not pregnant and had not delivered a child in the last 9 months, were nonsmokers, took no medications other than oral contraceptives and had no recent illness or vaccinations. Peripheral blood mononuclear cells were further processed to monocytes using the Monocyte Isolation Kit II (Miltenyi Biotec, San Diego, CA, USA). Monocytes were plated in a 96-well plate at 50 000 cells per well and rested overnight in complete RPMI media (10% fetal bovine serum, 1% L-glutamine). The next day, cells were treated with a monoclonal TLR4/MD-2 neutralizing antibody (mab-htlr4md2, InvivoGen) or control isotype antibody (mabg1-ctrlm, InvivoGen) at 20 μ g/mL, 1 hour before addition of stimulus. Cells were stimulated for 6 hours with LPS from *B. pseudomallei* K92643 at a final concentration of 1 ng/mL or heat-killed *S. aureus* at a bacteria to cell ratio of 100 before supernatants were collected. THP-1-Dual cells (InvivoGen), derived from the human THP-1 monocyte cell line, were differentiated over a period of 72 hours with vitamin D3 (final concentration 10 pM/mL) and plated at 50 000 cells per well in media containing D3. The next day, cells were transfected with Silencer Select siRNA (Ambion, Austin, TX, USA) against TLR4 (#4392420) or Silencer Select Negative Control No. 1 siRNA (#4390843) at a final concentration of 5 nM using Lipofectamine RNAiMAX Transfection Reagent (Life Technologies, Carlsbad, CA, USA). To evaluate efficiency of gene knockdown, total RNA was extracted from transfected cells after 48 hours using RNA lysis buffer (Promega). Extracted RNA was converted into cDNA using iScript Reverse Transcription Supermix (Bio-Rad, Hercules, CA, USA), according to the manufacturer's protocol. Expression of TLR4 and UBC (housekeeping gene) was quantified by quantitative real-time PCR using the SsoAdvanced Universal SYBRGreen Supermix (Bio-Rad) run on a Chromo4 (Bio-Rad). Each reaction was run in triplicate using 20 ng of template in a total volume of 20 μ L. Reactions were incubated at 95°C for 3 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds. PrimePCR primers purchased from Bio-Rad for TLR4, Human (qHsaCED0037607) and

UBC, Human (qHsaCED0023867) were used in the reactions. THP-1-Dual cells were stimulated 48 hours after siRNA transfection with either *B. pseudomallei* LPS 100 ng/mL as a positive control or heat-killed *S. aureus* at a bacteria-to-cell ratio of 100 at 37°C overnight. The following day, supernatants were collected.

Genotyping and single nucleotide polymorphism (SNP) selection

DNA was extracted from whole blood of healthy subjects and patients using the QIAamp DNA Blood Midi Kit (Qiagen). Selection of ninety six nonsynonymous coding SNPs in PRR pathway genes was performed by searching the HapMap project database (<http://hapmap.ncbi.nlm.nih.gov>) and based on functional prediction using a FastSNP analysis (<http://fastsnp.ibms.sinica.edu.tw>) [11]. Tag SNPs were selected from the Han Chinese in Beijing and Japanese in Tokyo populations in the HapMap database for variants with a minor allele frequency at least 2% using the HapMap tag-SNP picker option. Several inflammatory response SNPs previously associated with susceptibility or outcome from infection based on literature review were also included. Genotyping of healthy subjects was performed using Fluidigm SNPtype assays on a Biomark microfluidics real-time PCR system (Fluidigm, South San Francisco, CA, USA). rs1927907 was genotyped in patients using primers and probes designed by RealTimeDesign software from Biosearch Technologies (<https://www.biosearchtech.com>) and synthesized by Biosearch Technologies (Petaluma, CA, USA). The 147 bp region of TLR4 covering the variant position was amplified with forward primer 5'-GGTAGACCACCTCTCCCTTTT-3' and reverse primer 5'-GCTGGCCTCTCTGTAAGCA-3'. rs1927907 was genotyped using probes 5'-FAM-AGAAGTAGTTTTTACCAAACA-BHQ-1-3' for allele C and 5'-CAL Fluor Orange 560-CAAGAAGTAGTTTTTATCAAAC-BHQ-1 for allele T. Real-time PCR was performed using CFX96 Touch Real-Time PCR Detection System (Bio-Rad) in a total volume of 20 μ L.

Cytokine and chemokine measurements

Cytokine/chemokine assays were performed using Luminex multiplex bead system with R&D Systems reagents or enzyme-linked immunosorbent assay (BD; and R&D Systems, Minneapolis, MN, USA).

Statistical analyses

Continuous data following a normal distribution are reported as mean \pm standard deviation; continuous data following a non-normal distribution are reported as median \pm interquartile ranges. Plasma cytokine data were log₁₀ transformed before analysis, given their generally nonnormal distribution. For the immunoassay data set, cytokine correlations were assessed using a Pearson correlation coefficient, and correlation matrices were used to create heat maps [12] and relatedness dendrograms. To maximize power for identifying genetic associations, a composite cytokine response was created. The monocyte-normalized responses for five cytokines (interleukin (IL)-6, IL-8, tumor necrosis factor (TNF)- α , granulocyte-colony stimulating factor (G-CSF) and IL-1 β) were summed after subtracting the mean and dividing by the standard deviation of the 300 observations within each cytokine. These cytokines were chosen on the basis of the relatedness score in a cluster dendrogram. Linear regression of each SNP on composite cytokine response was performed assuming an additive genetic model, adjusting for log monocyte count. Eight of the 51 SNPs had observed minor allele frequencies of <0.05 and were removed from the primary analysis because of low power in the face of multiple test adjustment, leaving 43 SNPs for testing with the composite

cytokine outcome. Adjustment for multiple testing was done via permutation testing [13].

The distribution of the ordered p values under the null hypothesis was produced by permuting the outcomes 2000 times and recomputing the 43 p values for each permutation. The distribution of the minimum of the 43 p values for each of the permutations was compared with the observed minimum p value to obtain the probability of observing this minimum under the null hypothesis (i.e. a multiple-test corrected p value). Corrected p values for the second and third most significant SNPs (and so on) were obtained analogously. Associations were considered significant if the corrected p value was ≤ 0.05 . Further tests of association of candidate SNPs with individual cytokines were performed on \log_{10} -transformed cytokine data using linear regression, adjusting for log monocyte count, again assuming an additive genetic model. For monocyte/monocyte cell line experiments, data were analysed with the *t* test. For the clinical study, association of genotype with cytokine response or clinical outcome was performed with Fisher's exact test (for clinical outcomes) or with regression, adjusting for age, sex, comorbidity index and site. The comorbidity index is a 10-point score, with 1 each given for lung disease, heart disease, kidney disease, liver disease, neurologic disease, haematologic disease, autoimmune disease, cancer, diabetes and alcoholism. Secondary analyses were performed adjusting for bacterial species. Analyses were performed by Stata 11.2 software (StataCorp, College Station, TX, USA), or R (R Foundation for Statistical Computing, Vienna, Austria; <http://www.r-project.org/>).

Ethics

Ethical approval was obtained from the ethics committees of the following institutions: Faculty of Tropical Medicine, Mahidol University, Bangkok; Sunpasitthiprasong Hospital, Ubon Ratchathani; Udon Thani Hospital, Udon Thani; Khon Kaen Hospital, Khon Kaen; and Faculty of Medicine (Srinagarind Hospital), Khon Kaen University, Khon Kaen, Thailand; and the University of Washington, Seattle.

Results

S. aureus induces robust inflammatory responses in blood

To evaluate the inflammatory response to *S. aureus* in human blood, we assayed pro- and anti-inflammatory cytokines and chemokines in plasma after stimulation of fresh whole blood from 300 healthy individuals with two different concentrations of heat-killed *S. aureus* (Fig. 1A). As expected, *S. aureus* induced significant increases above baseline values for all mediators (IL-1ra, IL-1 β , IL-6, IL-8, IL-10, TNF- α , G-CSF and monocyte chemotactic protein (MCP)-1), with considerable interindividual variation in responses.

A human *TLR4* variant is associated with cytokine responses to *S. aureus*

We next analysed the association of candidate PRR pathway genetic variants with cytokine responses induced by *S. aureus*. We generated a composite cytokine response by performing pairwise comparisons between different cytokines produced from whole blood of each donor after activation with *S. aureus* and creating a cluster dendrogram based on intermediator relatedness scores (Fig. 1B). The most closely related mediators were IL-1 β , IL-8, G-CSF, IL-6 and TNF- α ($r = 0.6–0.9$). We selected these mediators to use as the composite measure. We genotyped 96 SNPs in candidate PRR pathway genes, selected as described in the Methods, in all 300 subjects. Five assays failed and 39 assays had no variation. One assay

was excluded because of a call rate of $<97\%$. The remaining 51 variants are listed in Supplementary Table S1. Eight variants had observed minor allele frequencies (MAF) of <0.05 and were excluded because of low power. Forty-three variants were tested for an

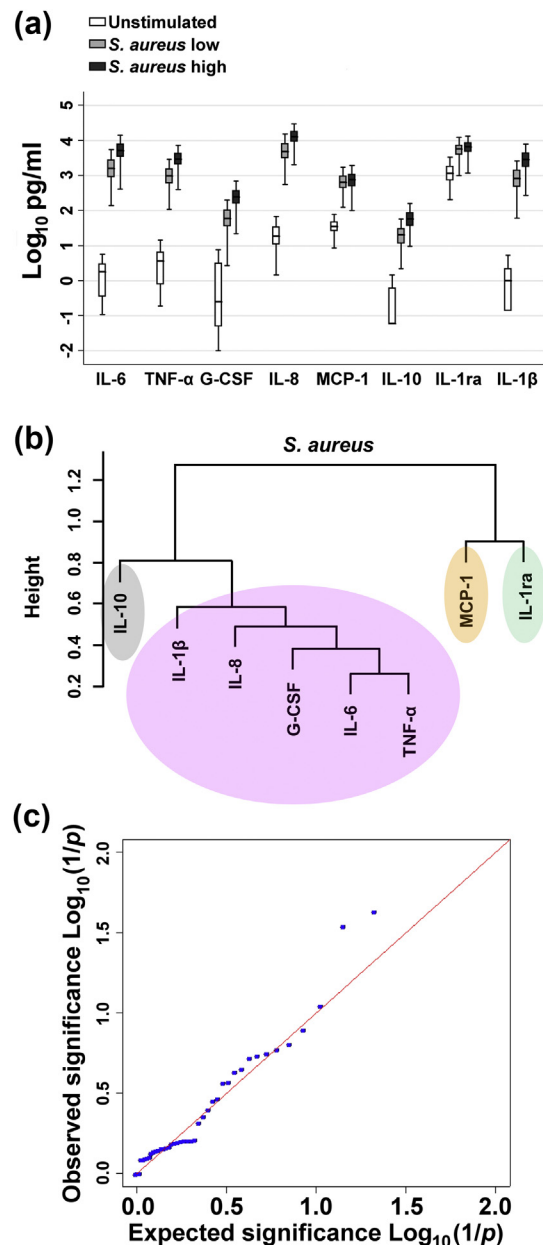


Fig. 1. SNP in *TLR4* is associated with *Staphylococcus aureus*-induced cytokine release. (a) IL-1ra, IL-1 β , IL-6, IL-8, IL-10, TNF- α , G-CSF and MCP-1 were measured in plasma supernatants by multiplex bead assay after stimulation of fresh whole blood from 300 healthy individuals with heat-killed *S. aureus* 2.5×10^7 CFU/mL (*S. aureus* low) or 5×10^7 CFU/mL (*S. aureus* high) for 6 hours at 37°C. Data are displayed on log scale. Boxes show median and interquartile range; whiskers show upper and lower adjacent values; outside values are not shown for clarity. All mediators induced by *S. aureus* are significantly greater than in unstimulated blood ($p < 0.001$ for all). (b) Cluster dendrogram created from relatedness scores calculated from pairwise comparisons between cytokines induced by stimulation of whole blood with *S. aureus* 2.5×10^7 CFU/mL used to derive composite outcome measure of TNF- α , IL-6, IL-8, G-CSF and IL-1 β responses. (c) QQ plot (expected vs. observed $\log_{10}(1/p)$ values) for association of 43 SNPs with composite outcome of *S. aureus*-induced cytokine levels demonstrating several lower-than-expected p values (higher-than-expected $\log_{10}(1/p)$ values). Lowest p value is rs1927907, intronic SNP in *TLR4*. G-CSF, granulocyte-colony stimulating factor; IL, interleukin; MCP, monocyte chemotactic protein; SNP, single nucleotide polymorphism; TNF, tumor necrosis factor.

association with cytokine response. We tested the association of each variant with the composite *S. aureus*–induced cytokine cluster (Fig. 1C). The top-ranked variant associated with cytokine response to *S. aureus* with $p < 0.05$ (adjusted for multiple comparisons via the permutation test) and a false discovery rate < 0.05 was rs1927907, an intronic *TLR4* polymorphism that exchanges a C for a T. The MAF of rs1927907 in our cohort was 0.10. To determine if a particular cytokine drove the observed composite effect, the association between rs1927907 and individual cytokine concentrations was tested. We found that four of the five cytokines induced by *S. aureus*—TNF- α , IL-1 β , IL-6 and G-CSF—were significantly higher for carriers of the rs1927907 minor (T) allele (Table 1). By comparison, rs1927907 was also associated with cytokine responses to *E. coli* LPS (TNF- α , IL-1 β , IL-6 and G-CSF) and Pam3CSK4 (TNF- α and IL-6), but not with cytokines induced by Pam2CSK4 or flagellin (data not shown).

rs1927907 is a *TLR4* expression quantitative trait locus (eQTL) in whole blood; the minor allele is associated with increased whole blood *TLR4* expression [14]. Moreover, the minor allele of rs1927907 is associated with increased *TLR4* protein expression on CD4⁺CD25^{high} regulatory T cells of asthmatic subjects as determined by flow cytometry [15]. These data, which are concordant with our observations of increased blood cytokine responses in carriers of the rs1927907 minor allele, implicate *TLR4* itself in the response to *S. aureus*. However, *TLR4* is the canonical LPS receptor and therefore is not anticipated to be involved in sensing of a Gram-positive pathogen. This prompted us to consider contamination of our *S. aureus* preparations with LPS as a potential explanation for these findings. We assayed our *S. aureus* preparations using a sensitive *Limulus* Amebocyte Lysate assay but did not detect any LPS (data not shown).

S. aureus does not activate *TLR4* in vitro

Because we did not find any LPS contamination in our preparations, we postulated that *S. aureus* might express other factors that would serve as ligands for *TLR4*. To address this question, we stimulated *hTLR4/MD-2/CD14*-transfected HEK293 cells with our *S. aureus* preparations to assess for a *TLR4*-dependent signal. We measured NF- κ B activation by luciferase reporter assay but found no *S. aureus*–induced *TLR4*-dependent light emission (Fig. 2A). This observation suggested that *S. aureus* does not express a *TLR4* ligand that signals through NF- κ B. We next evaluated how blockade of *TLR4* receptor function would affect cytokine responses to *S. aureus*. We stimulated primary blood monocytes from healthy subjects with *S. aureus* in the presence of a *TLR4/MD-2* neutralizing antibody and assayed TNF- α concentrations in cell supernatants (Fig. 2B). We observed no change in TNF- α release when *TLR4* receptor function was impaired. Together, these experiments indicated that *S. aureus* does not activate *TLR4* to induce inflammatory cytokine responses.

Knockdown of *TLR4* inhibits the TNF- α response to *S. aureus*

To explain our observations of differential cytokine response associated with rs1927907, we next evaluated whether

differential expression of *TLR4* in the cell may modify the host response to *S. aureus*. We surmised that even if *S. aureus* did not directly activate *TLR4*, there may be downstream modulatory effects of *TLR4* on other signaling pathways. We and others have observed such an effect of *TLR5* on *TLR4*-dependent signaling (Dickey AK, West TE, paper presented at the 2013 American Thoracic Society International Conference, abstract A4556; Collin CJ, Sciarba J, Rice A, Aloor J, Cyphert J, Bulek K, et al., paper presented at the 2016 American Thoracic Society International Conference, abstract A2643). We developed an assay using human monocyte THP-1-Dual cells in which we transfected siRNA targeting *TLR4* or control siRNA (Fig. 2C). We achieved approximately 50% decrease in *TLR4* gene expression in this assay. We next stimulated cells with *S. aureus* after transfection of siRNA against *TLR4* (Fig. 2D). We measured TNF- α concentration in cell supernatants and found that levels of this cytokine induced by *S. aureus* were significantly reduced after *TLR4* knockdown. Collectively, these results supported the concept that *S. aureus* does not directly activate *TLR4* but that *TLR4* modulates the inflammatory response to *S. aureus*.

TLR4 variant rs1927907 is associated with increased cytokine responses during staphylococcal sepsis

Regulators of the inflammatory response to heat-killed *S. aureus* in experimental settings may be very different than host response determinants in clinical staphylococcal infection. To determine whether rs1927907 might predict cytokine/chemokine responses in a clinical setting, we genotyped the variant in a cohort of 327 individuals with community-onset staphylococcal sepsis recruited at four hospitals in northeast Thailand. The clinical characteristics of these patients are described in Table 2. After finding no evidence for deviation from Hardy-Weinberg equilibrium in this cohort (p 0.61), we tested the association of rs1927907 with plasma cytokine concentration at enrolment. Minor allele homozygotes generated significantly higher levels of IL-6 and IL-8 in crude analyses and after adjusting for age, sex, comorbidity index and site (adjusted p 0.01 and 0.045, respectively) (Fig. 3).

TLR4 variant rs1927907 is associated with increased respiratory failure during staphylococcal sepsis

We then tested whether rs1927907 genotype predicted clinical outcome from staphylococcal sepsis. In both crude and adjusted analyses, minor allele homozygotes developed significantly more respiratory failure (adjusted odds ratio 9.65, 95% confidence interval 1.60–58.08, p 0.01) (Table 3). These individuals did not have significantly greater rates of shock or death, outcomes that were less frequent overall. Together, these cytokine and clinical data in an independent cohort of patients offer confirmatory evidence in support of the role of *TLR4* as a modulator of the host response in staphylococcal sepsis.

Table 1

Associations between *TLR4* variant rs1927907 and inflammatory mediators induced by *ex vivo* stimulation of blood from 300 healthy subjects with *Staphylococcus aureus*

rs1927907 genotype	Median (IQR) (pg/mL) for:				
	IL-6	IL-8	TNF- α	IL-1 β	G-CSF
CC	1480 (861–2656)	4532 (3090–7610)	927 (574–1433)	763 (463–1328)	55 (34–99)
CT	1852 (1304–4117)	5687 (3990–10956)	1128 (726–2684)	1100 (699–1727)	82 (49–148)
TT	3096 (701–5492)	7658 (2772–12544)	1833 (219–3447)	2243 (599–3886)	99 (27–170)
p^a	0.012	0.065	0.017	0.002	0.001

G-CSF, granulocyte-colony stimulating factor; IL, interleukin; IQR, interquartile range; TNF, tumor necrosis factor.

^a Linear regression model on log-transformed cytokine concentrations assuming an additive genetic model and adjusting for monocyte count.

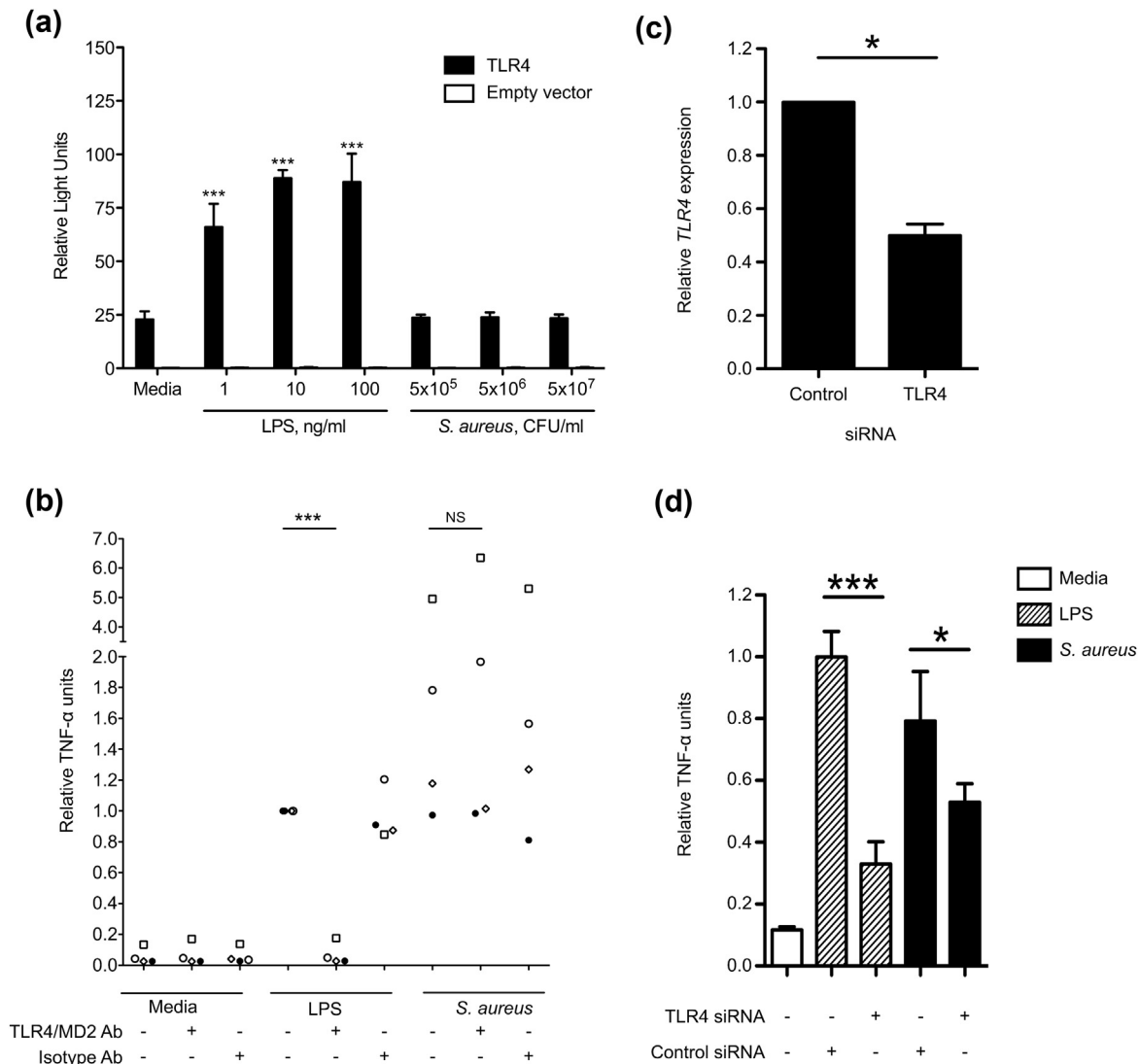


Fig. 2. *Staphylococcus aureus* does not activate NF- κ B or induce TNF- α release via TLR4 ligation but TLR4 modulates *S. aureus*-induced TNF- α release. (a) HEK293 cells were transiently transfected with *hTLR4* or equivalent amounts of empty vector, *hMD-2*, *hCD14*, ELAM (firefly luciferase) and pRL-TK (*Renilla* luciferase) before stimulation for 4 hours with *Escherichia coli* LPS or heat-killed *S. aureus*. NF- κ B activation was determined in cell lysates by ratio of firefly to *Renilla* luciferase light emission. Mean \pm standard deviation values of quadruplicate samples are displayed. *** p < 0.001 compared to unstimulated TLR4-transfected cells. (b) Primary blood monocytes from four healthy individuals were treated with monoclonal TLR4/MD2 neutralizing antibody or control isotype antibody at 20 μ g/mL, 1 hour before stimulation for 6 hours with LPS from *Burkholderia pseudomallei* K92643 1 ng/mL or heat-killed *S. aureus* at bacteria-to-cell ratio of 100. TNF- α was assayed by ELISA in supernatants. Individual data (each with unique symbol) representing means of duplicate or triplicate samples normalized to LPS-induced TNF- α release are displayed. *** p < 0.001. (c) THP-1-Dual cells were transfected with siRNA against TLR4 or negative control siRNA at 5 nM. After 48 hours, TLR4 gene expression was determined by quantitative real-time PCR. TLR4 gene expression is presented relative to housekeeping gene *UBC* expression and normalized to control siRNA transfection results. Mean \pm standard deviation values of quadruplicate samples from two independent experiments are displayed. * p < 0.05. (d) THP-1-Dual cells transfected for 48 hours as in (c) were stimulated with *B. pseudomallei* LPS 100 ng/mL or heat-killed *S. aureus* at bacteria-to-cell ratio of 100 overnight. TNF- α was assayed by ELISA in supernatants. Mean \pm standard deviation values of quadruplicate samples normalized to LPS-induced TNF- α release are displayed. Data comprise one representative example of three independent experiments. * p < 0.05; *** p < 0.001. ELISA, enzyme-linked immunosorbent assay; LPS, lipopolysaccharide; TNF, tumor necrosis factor.

Inflammatory responses to *S. aureus* correlate with responses to LPS and *B. pseudomallei*

In light of our data implicating TLR4 in the immune response to *S. aureus*, we then sought to determine whether inflammatory responses induced by *S. aureus* in healthy individuals might be correlated with responses induced by LPS or by Gram-negative bacteria in our human blood immunoassay. We selected *E. coli* LPS, *S. minnesota* LPS and heat-killed *B. pseudomallei* for this comparison. As control stimulants, we chose TLR2/6 agonist Pam2CSK4, TLR2/1 agonist Pam3CSK4, TLR5 agonist *S. typhimurium* flagellin, NOD1 agonist Tri-DAP and NOD2 agonist MDP. We measured the correlations between each stimulus and

created heat maps with derived relatedness dendrograms. Across all cytokines, the dendrograms indicated highest relatedness between responses to *S. aureus* and LPS or between *S. aureus* and *B. pseudomallei*. For example, the correlation between TNF- α induced by *S. aureus* and by *S. minnesota* LPS was 0.61 (p < 0.0001), and the correlation between IL-8 induced by *S. aureus* and by *B. pseudomallei* was 0.67 (p < 0.0001) (Fig. 4).

Discussion

The main findings of this study are as follows: blood inflammatory responses to *S. aureus* are associated with the TLR4 genetic polymorphism rs1927907, an eQTL, both *ex vivo* and in patients

Table 2

Clinical characteristics and outcomes of 327 patients with staphylococcal sepsis

Variable	Value
Age, years, median (IQR)	54 (42–65)
Female sex, n (%)	114 (34.9)
Preexisting conditions, n (%)	
Diabetes mellitus	116 (35.5)
Heart disease	17 (5.2)
Kidney disease	53 (16.2)
Liver disease	13 (4.0)
Lung disease	18 (5.5)
Cancer	7 (2.1)
Alcoholism	5 (1.5)
Autoimmune disease	5 (1.5)
Neurologic disease	8 (2.5)
Haematologic disease	9 (2.8)
Comorbidity index, median (IQR) ^a	1 (0–1)
Outcome, n (%)	
Respiratory failure	58 (17.7)
Shock	38 (11.6)
Death at 28 days	30 (9.2)

^a Comorbidity index is a 10-point score with 1 point each given for lung disease, heart disease, kidney disease, liver disease, neurologic disease, haematologic disease, autoimmune disease, cancer, diabetes and alcoholism.

with staphylococcal sepsis; TLR4 is not activated by *S. aureus* but *TLR4* knockdown impairs TNF- α release induced by *S. aureus*; rs1927907 predicts respiratory failure in patients with staphylococcal sepsis; and blood inflammatory responses to *S. aureus* are tightly correlated with responses to LPS and a Gram-negative organism. These findings raise intriguing new questions about the role of *TLR4* in human staphylococcal sepsis.

TLR4 is not usually considered a fundamental participant in the host response to Gram-positive infections. The *S. aureus* cell wall is composed of peptidoglycan in combination with other substances such as lipoteichoic acid (LTA) and lipoprotein. These components and secreted proteins can induce inflammation and may contribute to sepsis [4]. PRRs on host immune cells involved in recognition of *S. aureus* include TLR2 in combination with TLR1 or TLR6 and/or CD36 and CD14 [4]. While the cell wall of Gram-negative bacteria shares many of the same PAMPs as Gram-positive bacteria [3], a cell wall component unique to Gram-negative organisms is LPS [5]. LPS is known as a TLR4 agonist and induces a robust proinflammatory response and septic shock [16], but it is not expressed by *S. aureus*. Therefore, when we found associations between inflammatory responses and genetic variation in *TLR4* and observed tight correlation between responses to

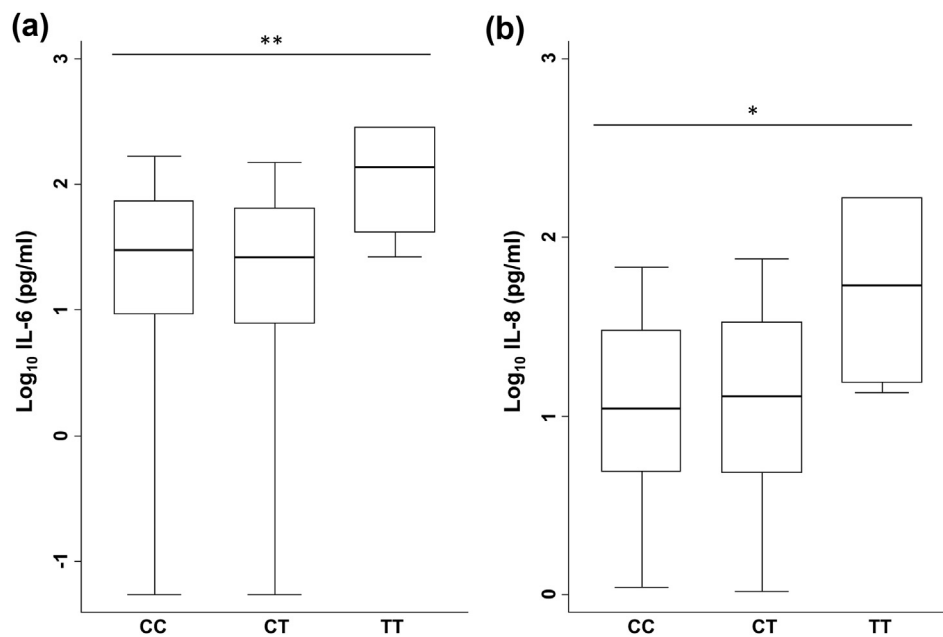


Fig. 3. *TLR4* variant rs1927907 is associated with plasma IL-6 and IL-8 in patients with staphylococcal sepsis. IL-6 (a) and IL-8 (b) were assayed by ELISA in plasma samples from patients with staphylococcal sepsis and are displayed by *TLR4* variant rs1927907 genotype. Boxes show median and interquartile range; whiskers show upper and lower adjacent values; outside values are not shown for clarity. Values were log₁₀ transformed, and linear regression was performed using recessive model (comparing CC and CT subjects to TT subjects), adjusting for age, sex, comorbidity index and site. *n* = 249 (CC), 69 (CT), 6 (TT). ***p* ≤ 0.01; **p* < 0.05. ELISA, enzyme-linked immunosorbent assay; IL, interleukin.

Table 3Association of *TLR4* variant rs1927907 with clinical outcomes of patients with staphylococcal sepsis

Genotype	Respiratory failure		Shock		Death	
	Yes	No	Yes	No	Yes	No
CC	42 (72%)	210 (78%)	29 (76%)	223 (77%)	22 (73%)	230 (77%)
CT	12 (21%)	57 (21%)	7 (18%)	62 (21%)	7 (23%)	62 (21%)
TT	4 (7%)	2 (1%)	2 (5%)	4 (1%)	1 (3%)	5 (2%)
Unadjusted <i>p</i> ^a	0.018		0.22		0.51	
Adjusted OR (95% CI)	9.65 (1.60–58.08)		4.31 (0.66–27.96)		1.75 (0.18–17.11)	
Adjusted <i>p</i> ^b	0.01		0.13		0.63	

CI, confidence interval; OR, odds ratio.

^a Unadjusted analyses performed by exact test.

^b Adjusted analyses performed by logistic regression assuming recessive genetic model adjusting for age, sex, comorbidity index and site.

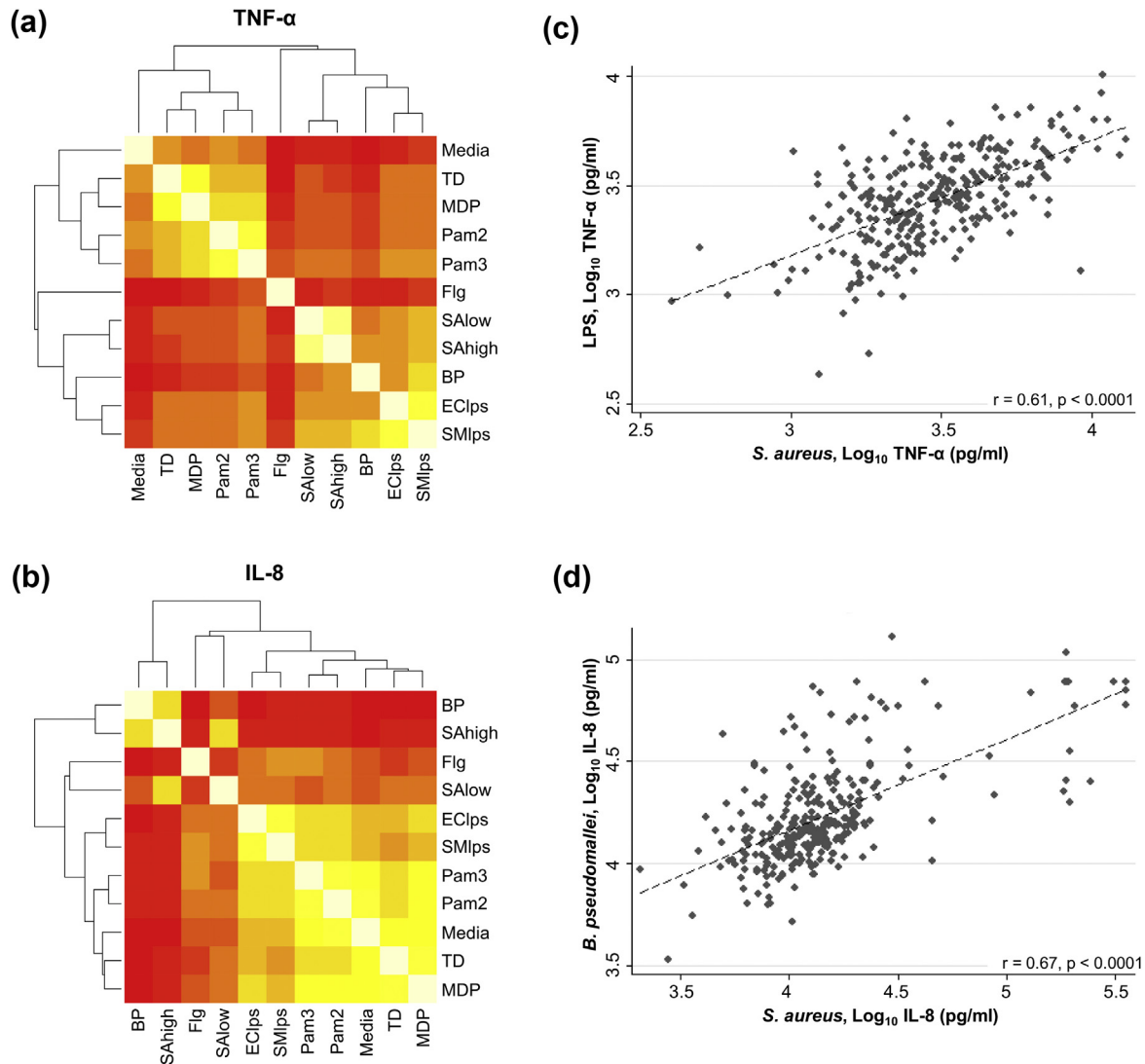


Fig. 4. High correlation between blood inflammatory responses induced by *Staphylococcus aureus* and LPS or *Burkholderia pseudomallei*. Heat maps of correlation between TNF- α (a) or IL-8 (b) responses induced by stimulation of whole blood from 300 healthy subjects with panel of purified innate immune ligands and heat-killed bacteria for 6 hours at 37°C. Stimulants were heat-killed *S. aureus* 2.5×10^7 CFU/mL (SALow) and 5.0×10^7 CFU/mL (SAhigh), heat-killed *B. pseudomallei* 2.5×10^7 CFU/mL (BP), *Salmonella typhimurium* flagellin 500 ng/mL (Flg), Pam3CSK4 100 ng/mL (Pam3), Pam2CSK4 100 ng/mL (Pam2), *Escherichia coli* O111:B4 LPS 10 ng/mL (ECips) and *Salmonella minnesota* Re595 LPS 10 ng/mL (SMlps). Dendrogram of hierarchical clustering results for correlation matrix for each mediator is shown. Lowest correlation is red (Pearson correlation coefficient $r < 0.1$) and highest correlation is white ($r = 1.0$). Scatter plots of individual TNF- α (c) or IL-8 (d) responses (\log_{10} transformed) after stimulation of fresh whole blood from 300 healthy subjects with heat-killed *S. aureus* 5×10^7 CFU/mL, *S. minnesota* Re595 LPS (C) or heat-killed *B. pseudomallei* 2.5×10^7 CFU/mL (d) for 6 hours at 37°C. For each plot, relationship between variables as determined by linear regression (dotted line) and Pearson correlation (correlation coefficient, r) are shown. IL, interleukin; LPS, lipopolysaccharide; TNF, tumor necrosis factor.

S. aureus and to LPS, we first excluded detectable LPS contamination in our experiments. We also considered that activation of TLR4 may occur by bacterial ligands other than LPS [17]. For example, the *S. aureus* exotoxin leukocidin has been reported to induce TLR4-dependent immune responses [18]. Yet we did not find that TLR4 was activated by our preparations of *S. aureus*, at least via the transcription factor NF- κ B.

Two other explanations may account for our findings. First, TLR4 may respond to endogenous ligands produced by the host [19]; during infection, this effect may be sufficiently strong to result in altered TLR4-dependent inflammatory responses and clinical outcomes. This is supported by a report that haemoglobin, a host blood product, can significantly enhance macrophage secretion and expression of several cytokines and receptors in a TLR4-dependent pathway when activated with low levels of TLR2 ligands such as LTA and Pam3CSK4. Haemoglobin can also lead to

secretion of high mobility group box 1 protein (HMGB1), which synergizes with LTA to increase secretion of IL-6 [20]. Second, TLR4 may contribute to the inflammatory response without direct ligation of the receptor. This seems like the most likely explanation for our observations, as cytokine release in monocytes was not impaired by antibody-mediated blockade of TLR4, yet cytokine release in a monocyte cell line was blunted after transfection of siRNA reducing TLR4 gene expression. Cooperative behavior by TLRs may alter inflammatory responses even when the ligand is specific for only one of the interacting TLRs. For example, we and others have found that TLR5 may alter TLR4-dependent signaling (Dickey AK, West TE, paper presented at American Thoracic Society International Conference, 2013, abstract A4556; Collin CJ, Scirba J, Rice A, Aloor J, Cyphert J, Bulek K, et al., paper presented at American Thoracic Society International Conference, 2016, abstract A2643). Furthermore, recent work suggests that TLR4

contributes to murine macrophage responses to *S. aureus* and that TLR2 and TLR4 may interact in this process [21]. Thus, although we present data that implicate *TLR4* as a modulator of the host response to *S. aureus*, the mechanism by which this may occur requires further elucidation.

Genetic variation in innate immunity is well established as a modulator of inflammatory responses and outcome in sepsis [22–24]. Our focus on Thai subjects offers important new data as population differences in genetic association studies of sepsis are critical to consider. For example, functional *TLR1* variants are associated with outcomes from sepsis in white North American subjects [22], but we have shown that *TLR1* genetic architecture is profoundly different and variants are nonfunctional in Southeast Asians [25]. rs1927907 is an intronic variant in *TLR4* that is common throughout the world (MAF 0.11–0.25 [26]). Notably, the clinical associations reported to date are restricted to Chinese populations; the variant is associated with late-onset Alzheimer disease [27], asthma severity [15,28] and tacrolimus pharmacokinetics after liver transplantation [29], neonatal early Crohn disease [30] and chronic periodontitis in a *TLR4* haplotype [31]. In the Southern Han Chinese population, the variant is in high linkage disequilibrium ($r^2 = 0.87$) with a *TLR4* promoter region variant, rs10983755 [26]. Ragnarsdottir *et al.* have reported that rs10983755 disrupts a potential binding site for the transcription factor N-Myc and increases promoter activity in HEK293 cells on infection with *E. coli* [32]. Moreover, rs1927907 is in high linkage disequilibrium with rs960312, a *TLR4* polymorphism that we have found to be associated with susceptibility to melioidosis, a common Gram-negative infection in northeast Thailand [33]. Thus, the haplotype tagged by rs1927907 is of considerable interest.

Several lines of evidence link rs1927907 to altered *TLR4* expression at both the gene and protein level. In a study of over 5300 whole blood specimens, rs1927907 was identified as a *TLR4* eQTL, with increasing *TLR4* gene expression observed in carriers of the minor allele [14]. Moreover, the minor allele of rs1927907 is associated with increased *TLR4* protein expression on CD4⁺CD25^{high} regulatory T cells of asthmatic subjects as determined by flow cytometry [15]. While not excluding *trans* (more distant) effects of rs1927907 on expression of other genes, these findings complement our findings of increased cytokine responses in carriers of the rs1927907 minor allele and more concretely implicate *TLR4* in this process. Further work is required to more completely understand the variation driving the observed associations and to replicate the clinical associations in other populations.

In light of the overlapping but not identical pathways activated by Gram-positive and Gram-negative bacteria, whether and how Gram-positive sepsis differs from Gram-negative sepsis is debated [34,35]. In comparison to other PAMPs, we found that responses to LPS and to *B. pseudomallei* were most highly correlated with *S. aureus*-induced responses. This suggests a shared common pathway of inflammation precipitated by Gram-positive and Gram-negative bacteria. A study by Tang *et al.* showed that the human sepsis transcriptome in Gram-positive sepsis is similar to the transcriptome in Gram-negative sepsis [36]. While this report also supports a common host response in the pathophysiology of sepsis regardless of infecting organism, a potentially confounding factor in clinical or *in vivo* studies is that LPS derived from gut bacteria may be released systemically during bowel hypoperfusion from sepsis of any etiology [37]. Our *ex vivo* data, obtained under controlled experimental conditions, avoid confounding by gut bacteria LPS release and provide corroborating protein-level evidence of shared pathway activation.

Several important limitations to our study deserve mention. First, the use of killed bacteria may not mimic the innate immune responses induced by live pathogens. Second, our analysis is

restricted to the optimal concentrations of specific PAMPs chosen that induced detectable cytokine responses; conceivably there may be additional dose-dependent effects that have not been identified. Third, our genetic analyses may be confounded by population stratification and may potentially differ in non-Thai subjects. Fourth, although we reported significant associations of rs1927907 with respiratory failure or plasma cytokines, the number of subjects with the recessive genotype in our clinical study was low, and our results may be biased by unmeasured confounding. While the association of rs1927907 with cytokine responses in whole blood stimulation *ex vivo* was determined using an additive genetic model, the associations in the clinical study were apparent using a recessive genetic model. This may be due to inadequate power to distinguish between the relative contributions of these models in human infection, or it may reflect different biological effects *ex vivo* versus in infected humans. Independent replication of these results in larger clinical cohorts is therefore important. Fifth, our study was likely underpowered to detect associations with shock or death, but we note that IL-6 and IL-8 levels strongly predict these outcomes in our cohort. Finally, the proinflammatory cytokines investigated in our study are mainly driven by shared NF- κ B/MAP kinase-signaling pathways downstream of the PRRs. We did not investigate interferon production, such as that mediated by interferon regulatory factors (IRFs). It has been recently reported that *S. aureus* RNA activates IRF5 via TLR8 and that this is antagonized by TLR2 activation [38]. How this sensing pathway relates to our findings necessitates further investigation.

Strengths of our immunoassay study include the prospective nature of healthy subject recruitment from a blood donor pool, the large number of subjects assayed and highly standardized blood processing techniques, as well as batched generation of assays, measurement of cytokines and genotyping. Furthermore, we replicated and extended our initial genetic associations in patients from the same region recruited in a prospective multisite study of staphylococcal sepsis. To our knowledge, our study is one of the first to identify innate immune genetic variants that predict outcomes from Gram-positive sepsis in Asians.

In conclusion, in Thais, a genetic variant in *TLR4* that is an eQTL is associated with whole blood cytokine responses to *S. aureus ex vivo* and with plasma cytokine levels and respiratory failure in staphylococcal sepsis. While *S. aureus* does not express LPS—the canonical *TLR4* ligand—or activate *TLR4* directly, the innate immune response to *S. aureus* does appear to be modulated by *TLR4* and shares significant commonality with that induced by Gram-negative pathogens and LPS. These data suggest a regulatory role for *TLR4* beyond canonical LPS-receptor ligation in Gram-positive sepsis.

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Transparency Declaration

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.cmi.2016.08.028>.

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