The Role of NOD2 in Murine and Human Melioidosis


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The Role of NOD2 in Murine and Human Melioidosis

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Nucleotide-binding oligomerization domain 2 (NOD2) is a cytosolic pathogen recognition receptor that regulates susceptibility to a variety of infections and chronic diseases. Burkholderia pseudomallei, a facultative intracellular bacterium, causes the tropical infection melioidosis. We hypothesized that NOD2 may participate in host defense in melioidosis. We performed a series of in vitro assays and in vivo experiments and analyzed the association of human genetic variation with infection to delineate the contribution of NOD2 to the host response to B. pseudomallei. We found that transfection with NOD2 mediated NF-κB activation induced by B. pseudomallei stimulation of HEK293 cells. After low-dose inoculation with aerosolized B. pseudomallei, Nod2-deficient mice showed impaired clinical responses and permitted greater bacterial replication in the lung and dissemination to the spleen compared with wild-type mice. IL-6 and KC levels were higher in the lungs of Nod2-deficient mice. In a cohort of 1562 Thai subjects, a common genetic polymorphism in the NOD2 region, rs7194886, was associated with melioidosis, and this effect was most pronounced in women. rs7194886 was not associated with differences in cytokine production induced by whole-blood stimulation with the NOD2 ligand, muramyl dipeptide, or B. pseudomallei. To our knowledge, these findings are the first to characterize the role of NOD2 in host defense in mammalian melioidosis. The Journal of Immunology, 2014, 192: 300–307.

Melioidosis is a severe infection caused by the soil saprophyte Burkholderia pseudomallei. Endemic in parts of southeast Asia and northern Australia, the disease is often characterized by severe sepsis, indicative of a dysregulated host immune response (1). Pneumonia is a common manifestation of disease, either secondarily due to hematogenous spread, or due to primary inhalation of bacteria (1).

TLRs, membrane-associated pathogen recognition receptors, have already been implicated in governing the host response in murine and human melioidosis (2–5). TLR2, a sensor of lipopeptides, is deleterious in mice infected with B. pseudomallei via the intranasal route, an effect that is most apparent several days following infection (4). TLR4 recognizes LPS and contributes to bacterial containment in the first 24 h of murine respiratory infection with B. pseudomallei or with the related organism Burkholderia thailandensis, but has no effect on mortality (2, 4, 6). However, human genetic polymorphisms in TLR4 are associated with susceptibility to melioidosis (3). In patients with melioidosis, a nonsense polymorphism in TLR5, a flagellin sensor, is associated with survival (5). A complementary set of pathogen recognition receptors, the nucleotide-binding oligomerization domain (NOD)–like receptors (NLRs), exists in the cytosol. These proteins contain an N-terminal caspase and recruitment domain (CARD) and a central leucine-rich repeat. NLRs can be broadly divided into the following: 1) those (such as NLRC4) that contribute to the assembly of the inflammasome, a molecular platform that permits caspase-1 activation, and 2) the noninflammasome NLRs, such as NOD1 and NOD2 (7). NOD2 is expressed in monocytes, macrophages, dendritic cells, intestinal Paneth cells, and lung epithelial cells (8, 9). Upon ligation of bacterial cell wall component muramyl dipeptide (MDP) by NOD2, CARD proteins RIP2/RICK and CARD9 are recruited and signaling occurs via MAPK and NF-κB pathways (10). Because of the cytosolic location of NLRs, they may function synergistically with TLRs to promote a proinflammatory state (11).

B. pseudomallei is a facultative intracellular pathogen that readily escapes from endosomes into the cytosol (12), highlighting the potential importance of NLRs and related cytosolic signaling pathways in host defense in melioidosis. In mice, the type III secretion system of B. pseudomallei is detected by NLRC4 and resistance to murine melioidosis requires both NLRC4 and caspase-1 (13–15). B. pseudomallei upregulates NOD2 expression...
in a mouse macrophage cell line (16), but otherwise the function of NOD2 in melioidosis is largely unknown. Given the established function of specific TLRs in melioidosis and potential for synergistic effects of cytosolic receptors, we hypothesized that NOD2 may play a role in modulating host defense in melioidosis. To test this hypothesis, we examined the role of NOD2 in regulating innate immune responses to B. pseudomallei in vitro and in a murine model of respiratory melioidosis, and we tested the association of human genetic NOD2 polymorphisms with disease in a cohort of Thai subjects.

**Materials and Methods**

**Bacteria**

*B. pseudomallei* 1026b (17) was used for all experiments. Heat killing was accomplished by growing bacteria for 6 h shaking at 180 rpm at 37°C in Luria–Bertani (LB) broth (2). Bacteria were washed twice in sterile PBS and resuspended in PBS before being heat killed for 45–60 min at 65°C. Bacterial concentration and confirmation of successful killing were determined by quantitative culture. For in vivo studies, *B. pseudomallei* 1026b was grown in LB broth shaking in air at 37°C, washed twice, resuspended in PBS containing 20% glycerol, and frozen at −80°C. Immediately before each aerosol infection experiment, the freezer thawed and diluted in PBS to the desired concentration, as previously described (18).

**Transfections**

HEK293 cells were seeded at 50,000 cells/well in a 96-well flat-bottom tissue culture plate to reach at least 90% confluency at the time of transfection. After 2 d, cells were simultaneously transfected and stimulated. Cells were transfected using FuGENE HD (Roche, Mannheim, Germany) at a ratio of 6 μl FuGENE HD per 2 μg DNA according to the manufacturer’s instructions. Vector DNA transfected consisted of 10 ng/well NF-kB-ELAM firefly luciferase, 1 ng/well control pRL-TK Renilla luciferase (Promega, Madison, WI), and 0.1 ng/well either empty vector or huNOD2-WT-pEF6 (19). After transfected DNA was applied to all wells, cells were immediately stimulated in triplicate with media, control NOD2 ligand MDP (Invivogen, San Diego, CA), or heat-killed bacteria. Cells were incubated overnight. The following day, cells were washed three times with Dulbecco’s PBS and lysed with 20 μl/well passive lysis buffer included with dual-luciferase reporter assay system (Promega). Activation of NF-kB was determined in 10 μl/well cell lysate using the dual-luciferase reporter assay system and a Veritas Microplate Luminometer (Turner Biosystems/Promega, Sunnyvale, CA).

**Mouse model of melioidosis**

**Animals.** Specific pathogen-free C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Nod2−/− mice backcrossed eight generations onto a C57BL/6 background are previously described (19). Mice were housed in isolator cages with ad lib access to chow and water, and were monitored one to two times daily. All animal experiments were approved by the University of Washington Institutional Animal Care and Use Committee.

**Infection.** Mice were exposed to aerosolized bacteria in a 24-port cylindrical nose-only exposure chamber (In-Tox Products, Moriarty, NM) (18). Aerosols were generated by a MiniHeart Hi-Flo nebulizer (Westmed, Tucson, AZ) driven at 8 L/min with 7 L/min simultaneous dilution air for 10 min, followed by a 5 min washout period. Pressure and airflow were controlled by an AeroMP aerosol management platform (Baiera Technologies, Frederick, MD). Bacterial deposition in each experiment was determined from a nose-only exposure chamber (In-Tox Products, Moriarty, NM) (18). Aerobic deposition in each experiment was determined from the manufacturer’s instructions. Vector DNA transfected consisted of 10 ng/well NF-kB-ELAM firefly luciferase, 1 ng/well control pRL-TK Renilla luciferase (Promega, Madison, WI), and 0.1 ng/well either empty vector or huNOD2-WT-pEF6 (19). After transfected DNA was applied to all wells, cells were immediately stimulated in triplicate with media, control NOD2 ligand MDP (Invivogen, San Diego, CA), or heat-killed bacteria. Cells were incubated overnight. The following day, cells were washed three times with Dulbecco’s PBS and lysed with 20 μl/well passive lysis buffer included with dual-luciferase reporter assay system (Promega). Activation of NF-kB was determined in 10 μl/well cell lysate using the dual-luciferase reporter assay system and a Veritas Microplate Luminometer (Turner Biosystems/Promega, Sunnyvale, CA).

**Lung histology and quantitative morphometry.** The right lung was fixed in 4% paraformaldehyde, as previously described (18). Lung tissue was embedded in paraffin, sectioned, and stained with H&E; sections were examined by a veterinary pathologist who was blinded to group assignment. The number of foci inflammatory lesions, area of each lesion, and total tissue area in one representative section from each mouse were determined using Nikon NIS-Elements software.

**Cytokine measurements.** Left lung homogenates in PBS were diluted 1:1 in lysis buffer containing 2 × protease inhibitor mixture (Roche Diagnostics, Mannheim, Germany), incubated on ice for 30 min, and then centrifuged at 1500 × g. Supernatants were collected and stored at −80°C until assayed for cytokines. Whole blood was centrifuged, and serum was removed and stored at −80°C until assayed. IFN-γ, IL-10, IL-12p70, IL-1β, IL-6, KC, and TNF-α were measured in lung homogenates and serum using a 7-plex electrochemiluminescence detection assay (Meso Scale Discovery, Gaithersburg, MD) and read on the MSD Sector Imager 2400.

**Human subjects**

**Clinical cohort.** Human genetic analyses were performed on 614 inpatients with culture-proven melioidosis admitted to Sappasithiprasong Hospital (Ubon Ratchathani, Thailand) from July 1999 through December 2005, and 950 ambulatory control subjects recruited at the hospital through 2010. Subsets of this cohort have been described previously (3, 5). DNA was extracted from blood using a Nucleon BACC3 kit (GE Healthcare, Buckinghamshire, U.K.). Consent for enrollment into clinical studies of melioidosis was obtained from subjects or their representatives at the time of recruitment.

**Ex vivo blood stimulation.** Three hundred Thai subjects donating blood at the blood donation center at Sappasithiprasong Hospital were recruited for a blood sample, as previously described (5). Subjects were included if they indicated that they were between the ages of 18 and 60 y and did not report any history of immunodeficiency or inflammatory conditions, chronic diseases, pregnancy in the past 6 mo, anti-inflammatory medication use in the past week, antibiotic use in the past 24 h, heavy exercise or alcohol consumption in the past 24 h, or smoking in the past month. Those who met enrollment criteria gave written informed consent to participate and provided a postdonation blood sample in citrate tubes. A complete blood count with differential was determined for each subject in the central laboratory. A batch of 96-well immunoassay plates was generated by adding 20 μl innate immune ligands and heat-killed bacteria in appropriate concentrations to each well. Plates were frozen at −80°C until the day of use, when they were thawed to 37°C. A total of 380 μl fresh whole blood anticoagulated with citrate from each subject was mixed 1:1 with RPMI 1640 media and added to each well. For this study, the stimulants analyzed were 10 μg/ml MDP (Invivogen) and heat-killed *B. pseudomallei* 1026b. Plates were placed on a shaker at 37°C under 5% CO2 for 6 h before being spun down, and the plasma supernatant was removed and frozen at −80°C. Cytokine concentrations were subsequently assayed using R&D Systems reagents on a Luminex multiplex bead system. For this study, the analysis was restricted to IL-6, TNF-α, IL-1β, and IL-10. Due to out-of-range values in the multiplex assay, IL-1β concentrations for *B. pseudomallei* were determined by ELISA (BD Biosciences). DNA was extracted from whole blood using the QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany).

**Human subjects.** The University of Washington Human Subjects Division Institutional Review Board; the Ethical Review Committee for Research in Human Subjects, Ministry of Public Health, Thailand; the Ethical Review Committee for Research in Human Subjects, Sappasithiprasong Hospital; and the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand, approved the studies.

**Polymorphism selection and genotyping**

Human single-nucleotide polymorphisms in the NOD2 gene region that are associated with Crohn’s disease or leprosy were identified from the published literature. The frequency of these variants in Asian populations was determined using the National Center for Biotechnology Information dbSNP database and the Genome Variation Server (http://gvs.gs.washington.edu/GVS/). Seven common variants were selected for assay design and analysis. Genotyping was performed using ABI TaqMan assays on an ABI Prism 7900 (Carlsbad, CA).

**Statistics**

Comparisons of two groups of data expected to follow a normal distribution were made using Student t test. CFUs were log_{10} transformed before analysis. Counts of murine signs of illness were compared using a test of
We measured NF-κB activation by cotransflecting a NF-κB-ELAM-luciferase construct and control thymidine kinase *Renilla* luciferase construct, and quantifying relative light units. In NOD2-transfected cells, we observed a significant increase in NF-κB activation induced by *B. pseudomallei*, indicating recognition of *B. pseudomallei* motifs by NOD2.

**Nod2 deficiency impairs the clinical response in low-dose murine respiratory melioidosis**

We then examined the effect of *Nod2* deficiency in a murine model of respiratory melioidosis. We chose this model because inhalation is a route of infection in human melioidosis and because pneumonia is one of the most common manifestations of severe disease. We have developed a reproducible model of murine pneumonia by aerosolizing *B. pseudomallei* to mice (18). In this model, we have found that the median lethal deposition dose to C57BL/6/mice is 334 CFU/lung. However, there is a narrow range for lethality as all C57BL/6 mice infected with 292 CFU/lung survive, whereas 100% of mice infected with 375 CFU/lung die.

We conducted several experiments to test whether *Nod2* deficiency altered survival from melioidosis. We deposited 92 CFU *B. pseudomallei* per lung by aerosol to wild-type C57BL/6 and *Nod2*-deficient mice, and monitored survival. At this dose, all wild-type mice survived, but only two of five *Nod2*-deficient mice survived (*p* = 0.05 by the log-rank test) (Fig. 2).

In a second experiment at a marginally higher dose, we deposited 134 CFU/lung to wild-type and *Nod2*-deficient mice (Fig. 2). All eight mice in each group survived for duration of the experiment (*p* = 1.0). Although this experiment did not replicate the survival advantage of *Nod2* seen previously, there were clear differences in clinical condition between mouse strains (Table I). Following infection, the wild-type mice remained clinically well for the entire 11 d clinical monitoring period. In contrast, *Nod2*-deficient mice showed overt signs of illness manifesting primarily as coat scruffiness and hunching beginning at day 1 following infection. By day 2, all eight *Nod2*-deficient mice were scruffy and hunched, whereas no wild-type mouse showed any clinical signs of infection. This difference persisted until the end of the first week when the *Nod2*-deficient mice began to show clinical improvement.

In a third experiment targeting an even higher inoculation, we deposited 375 CFU/lung to wild-type and *Nod2*-deficient mice (Fig. 2). Infection was lethal to all the wild-type and three of four *Nod2*-deficient mice within 5 d, without any clear separation of survival curves (*p* = 0.75). Collectively, these experiments suggested that NOD2 does not clearly augment survival in murine melioidosis, especially at higher doses. For infection with lower doses of *B. pseudomallei*, however, NOD2 imparts detectable clinical benefit. To maximize our sensitivity to detect pertinent...
 phenotypes, we therefore targeted lower inoculating doses in subsequent experiments.

**Nod2 deficiency permits greater bacterial replication and dissemination in sublethal murine respiratory melioidosis**

To test whether NOD2 facilitates bacterial containment in murine respiratory melioidosis, we elected to aerosolize a low deposition dose of *B. pseudomallei* to wild-type and *Nod2*-deficient mice and quantify bacterial burdens in the lung and spleen. In wild-type C57BL/6 mice, we have observed that a deposition dose of 56 CFU/lung results in significant replication (up to 10^4 CFU) in the lung and dissemination to the liver and spleen by 24 h following infection (18). There is continued increase in bacterial burdens and associated inflammation from 24 to 48 h following sublethal infection that diminishes by 96 h (18). To identify maximal NOD2-dependent differences after low-dose infection, we therefore deposited 57 CFU *B. pseudomallei* in the lungs of wild-type and *Nod2*-deficient mice and quantitated bacterial burdens in the lung and spleen 48 h postinfection (Fig. 3). There was pulmonary replication and dissemination to the spleen in both groups of mice, but significantly more in *Nod2*-deficient mice compared with wild-type *Nod2*-deficient mice and wild-type mice 48 h postinfection (Fig. 3). There was continued increase in bacterial burdens and pulmonary cytokine release in the absence of *NOD2* after low-dose airborne infection in numerous studies and offers insight into the importance of specific elements of human host defense (20). We hypothesized that genetic variation in *NOD2* may be associated with melioidosis in a cohort of Thai subjects (3, 5). To test this, we examined the published literature for *NOD2* region single-nucleotide polymorphisms that had been previously associated with disease. Although nonsynonymous *NOD2* variants are associated with Crohn’s disease (21), none are common in Asian populations (minor allele frequency ≤1%). Other *NOD2* region single-nucleotide polymorphisms have been associated with leprosy or its clinical outcomes in Nepalese or Chinese populations (22–24). We chose seven candidate polymorphisms from these populations to genotype in our Thai cohort of 612 culture-confirmed melioidosis cases and 950 ambulatory controls.

**A common NOD2 region genetic variant is associated with melioidosis**

Our in vitro and murine studies supported a modest role for NOD2 as a contributor to the host response to *B. pseudomallei* infection under experimental conditions. However, we sought additional evidence for a role in *NOD2* in human melioidosis. Human genetic variation in innate immune genes has been associated with infection in numerous studies and offers insight into the importance of specific elements of human host defense (20). We quantified the average size of the inflammatory foci and determined the ratio of total foci area to total lung area (Fig. 5). We did not detect any differences in these measures between wild-type and *Nod2*-deficient mice, demonstrating that the greater bacterial burdens and pulmonary cytokine release in the absence of *NOD2* were not associated with any increase in histologically apparent inflammation.

**FIGURE 3.** *Nod2* deficiency permits greater bacterial replication and dissemination in murine respiratory melioidosis. Wild-type and *Nod2*-deficient mice were infected with 57 CFU/lung aerosolized *B. pseudomallei* 1026b. Lungs and spleens were harvested and quantitatively cultured 48 h postinfection. Data displayed are means ± SD and represent one experiment. *p ≤ 0.05. **p ≤ 0.001 for difference between mouse strains for each condition.

**WT, Wild type.**

Table I. Murine clinical responses in respiratory melioidosis

<table>
<thead>
<tr>
<th>Condition</th>
<th>Mouse Strain</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 7</th>
<th>Day 11</th>
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<td>Impaired mobility</td>
<td>WT</td>
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<td>Coat scruffiness</td>
<td>WT</td>
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*p ≤ 0.05. **p ≤ 0.001 for difference between mouse strains for each condition.
Five assays were successful (rs2287195, rs9302752, rs7194886, rs751271, and rs1077861) with a call rate of at least 97.5%. In the control subjects, linkage disequilibrium, a measure of nonrandom allelic association of different linked polymorphisms (r²), ranged from 0.07 to 0.97 for these variants. We confirmed the lack of deviation from Hardy Weinberg equilibrium for each variant in the controls and analyzed the genotype frequencies in cases and controls using an additive genetic model adjusted for age, gender, and diabetes (Table II). We did not apply a conservative Bonferroni correction given the observed linkage disequilibrium. We found that carriage of the minor T allele at rs7194886 was associated with melioidosis (odds ratio [OR] 1.32, 95% confidence interval [CI] 1.03–1.70, \( p = 0.029 \)). rs7194886 is located ∼5.8 kb upstream of NOD2 and has a minor allele frequency of 0.08 in control subjects. The magnitude of effect was stronger in a recessive model (OR 3.60, 95% CI 1.07–12.16, \( p = 0.039 \)). Unexpectedly, we observed a substantial effect of gender on the association (likelihood ratio \( p = 0.01 \); Supplemental Table I). For females, the OR for melioidosis in carriers of the variant was 12.56, 95% CI: 1.53–102.97, whereas for males the OR was 0.41, 95% CI 0.04–4.03. These data, although limited to an association, imply that NOD2 may mediate susceptibility to human melioidosis.

We next sought to determine whether rs7194886 has a demonstrable functional effect on the inflammatory response to \textit{B. pseudomallei}. We stimulated whole blood from 127 healthy Thai women with the NOD2 agonist MDP or heat-killed \textit{B. pseudomallei}. We measured IL-6, TNF-\alpha, IL-10, and IL-1\beta responses in plasma (Fig. 6). Cytokine responses to 10 mg/ml MDP were several orders of magnitude lower than responses induced by \textit{B. pseudomallei}, but were nonetheless readily detectable. We found no differences in cytokine concentrations based on rs7194886 genotype. Thus, in this assay, we could not ascribe a functional effect to this polymorphism.

**Discussion**

We show in this work that NOD2 activation by \textit{B. pseudomallei} induces activation of the innate immune transcription factor NF-kB; that the absence of NOD2 does not alter survival but has a modest effect on clinical response, bacterial containment, and cytokine release in the murine lung after low-dose infection with \textit{B. pseudomallei} in vivo; and that human \textit{NOD2} region genetic variation is associated with susceptibility to melioidosis. To our knowledge, this is the first study of the role of NOD2 in \textit{B. pseudomallei} infection in vivo and in humans, advancing our understanding of mammalian host defense to this facultative intracellular pathogen.

A growing body of literature points to the importance of NOD2 in facilitating the host response to experimental bacterial infections, yet the role of this receptor is infection specific (23, 25–29). For example, in murine pneumonia caused by \textit{Escherichia coli} or \textit{Chlamydia pneumoniae}, NOD2 contributes to bacterial containment in the lung (25, 26). However, in murine pneumonia due to \textit{Staphylococcus aureus} or \textit{Legionella pneumophila}, NOD2 is
redundant for this purpose (19, 28, 30). Our data indicate that, whereas there is no clear NOD2-dependent effect on murine survival after lethal or sublethal *B. pseudomallei* infection, NOD2 facilitates pulmonary containment of *B. pseudomallei* after low-dose infection. In the absence of NOD2, our results show that bacterial replication and dissemination are enhanced and are accompanied by clinical signs of illness and increased production of low levels of IL-6 and KC in the lung. Interestingly, this cytokine

### Table II. Association of NOD2 variants with melioidosis

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotype</th>
<th>Control</th>
<th>Case</th>
<th>HWE p Value</th>
<th>OR (95% CI)</th>
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<td>11</td>
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</table>

*a* Additive genetic model adjusting for age, gender, and diabetes. HWE, Hardy Weinberg equilibrium.

![FIGURE 6](http://www.jimmunol.org/) Whole-blood cytokine responses to NOD2 agonists by rs7194886 genotype. Monocyte-normalized plasma IL-6, TNF-α, IL-1β, and IL-10 levels induced by stimulation of whole blood from 127 healthy female subjects at 37°C for 6 h with medium alone, 10 μg/ml MDP (Invivogen), and heat-killed *B. pseudomallei* 1026b 2.5 × 10⁶ CFU/ml. Boxes show the median and interquartile range; whiskers show upper and lower adjacent values. n = 111 (CC), 15 (CT), 1 (TT). p > 0.05 for all comparisons between genotypes.
profile differs from *E. coli* pneumonia, in which *Nod2*−/− cytokine responses are blunted despite augmented bacterial counts in the lung (25). This difference may be explained by *B. pseudomallei*’s behavior as a facultative intracellular pathogen. Recently, Pudla et al. (16) showed that NOD2 knockdown impairs expression of the negative cytokine signaling regulator suppressor of cytokine signaling 3 in *B. pseudomallei*-infected macrophages. Together, these data imply that the mechanism by which NOD2 contributes to containment in murine melioidosis is not due to lack of cytokine production. Recent work indicates that NOD2 may control intracellular pathogens by facilitating autophagy (31), a recognized suppressor of *B. pseudomallei* survival in mammalian cells (32). This mechanism should be investigated in future studies.

Human genetic variation in NOD2 is well described, and NOD2 variation is associated with inflammatory disorders such as Crohn’s disease (21), Blau syndrome (33), sarcoidosis (34), asthma, and chronic obstructive pulmonary disease (35, 36). rs7194886 is an intergenic variant upstream of NOD2 that is associated with leprosy in a genome-wide association study in Chinese subjects and with type I reversal reactions and erythema nodosum leprosum in a candidate gene analysis in Nepalese subjects (22, 23). rs7194886 is not associated with leprosy in Vietnamese subjects (24); however, the variant is associated with tuberculosis in Chinese individuals (37). Thus, there is increasing evidence of its importance in mycobacterial infection in Asian populations. As such, its association with susceptibility to melioidosis—another intracellular infection that shares a number of clinical and pathophysiologic similarities with tuberculosis (38)—is quite plausible. Interestingly, the 1000 Genomes Project shows that the minor allele frequency of the variant is 0.45 in Europeans, 0.27 in Americans, 0.24 in Africans, but only 0.09 in East Asians (39). This suggests that there may be some selective pressure against the variant that is particularly pronounced in Asian populations. The effect of rs7194886 on NOD2 function is presently unknown; rs7194886 scores low as a regulatory variant in the RegulomeDB (40), but may lie within a haplotype with regulatory function. Whereas our ex vivo blood stimulation assay did not reveal genotype-dependent differences in cytokine responses induced by MDP or by killed *B. pseudomallei*, putative differences in NOD2-dependent autophagy would not be detected in this assay. Thus, although intriguing, our association of rs7194886 with melioidosis does not indicate causation. Our ongoing investigations are focused both on elucidating the architecture of variation in the NOD2 gene region in Asians and on identifying functional effects that may underlie the association of rs7194886 with disease.

Our observation of a gender-specific effect of a NOD2 region genetic variant is of particular interest. To date, few studies have examined the role of gender in governing the association of host genetic makeup with infectious disease. Several authors have reported effects of gender in genetic studies of lipids, obesity, or cardiovascular disease (41–44). It is possible that an unmeasured confounder may account for the gender-dependent genetic association in the case-control cohort. To our knowledge, gender has not been previously implicated in NOD2-dependent signaling, although gender-related differences in innate immune activation are well described (45–47). Our findings prompt further evaluation of the role of gender in modulating innate immune function.

An important consideration is the possibility of population admixture confounding our genetic association results. In previous analyses of a large subset of this cohort, however, we have not identified significant population stratification (3). Validation of genetic associations in independent populations is also desirable, although to our knowledge there are few other cohorts of melioidosis patients of sufficient size to permit this.

Together, our data comprise the most comprehensive investigation to date on NOD2 as a contributor to host defense in melioidosis and add to the expanding literature implicating cytosolic pathogen recognition receptors in the host response to intracellular infection.

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**Disclosures**
The authors have no financial conflicts of interest.

**References**

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Supplemental Table I: Interaction of gender with rs7194886 genotype in predicting melioidosis

<table>
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<tr>
<th>Model</th>
<th>Predictor a</th>
<th>Odds Ratio</th>
<th>95% CI</th>
<th>p value</th>
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<tr>
<td>1</td>
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<td>3.60</td>
<td>1.07-12.16</td>
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<td>Age</td>
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<td>1.01-1.02</td>
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<tr>
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<td>Diabetes</td>
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<td>0.70-1.15</td>
<td>0.37</td>
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<td>0.027</td>
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</tbody>
</table>

likelihood ratio test for difference between models p=0.012

a Recessive genetic model for rs7194886