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Toll-like Receptor (TLR) 2 Mediates Inflammatory Responses to Oligomerized RrgA Pneumococcal Pilus Type 1 Protein*

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Background: The pneumococcal pilus is associated with increased inflammation.

Results: A 49-amino acid region of the pilus protein RrgA activates TLR2 and is associated with increased inflammation and virulence.

Conclusion: The pneumococcal pilus is a TLR2 agonist; RrgA is a key component.

Significance: A better understanding of the pilus in bacterial pathogenesis is crucial for the development of novel strategies against this pathogen.

The pneumococcal type 1 pilus is an inflammatory and adherence-promoting structure associated with increased virulence in mouse models. We show that RrgA, an ancillary pilus subunit devoid of a lipidation motif, particularly when presented as part of an oligomer, is a TLR2 agonist. The surface-exposed domain III, and in particular a 49-amino acid sequence (P3), of the protein is responsible for the TLR2 activity of RrgA. A pneumococcal mutant carrying RrgA with a deletion of the P3 region was significantly reduced in its ability to activate TLR2 and induce TNF- α responses after mouse intraperitoneal infection, whereas no such difference could be noted when TLR2^{-/-} mice were challenged, further implicating this region in recognition by TLR2. Thus, we conclude that the type 1 pneumococcal pilus can activate cells via TLR2, and the ancillary pilus subunit RrgA is a key component of this activation.

Streptococcus pneumoniae (pneumococcus) accounts for over 860,000 childhood deaths annually (1). The bacterium is a frequent colonizer of the nasopharynx in children; most are colonized by the pneumococcus at some point during the first 2 years of life. Currently available pneumococcal vaccines generate potent systemic immunity via the generation of opsonophagocytic antibodies to the capsular polysaccharide (2, 3). These vaccines, however, have limitations, including selective coverage of capsular types included in the vaccines, only partial protection against mucosal disease, the phenomenon of serotype replacement, and high cost (4–6). For these reasons,

alternative vaccine strategies have been sought, including the development of multivalent protein-based vaccines or killed whole cell vaccines (7–12).

Many surface proteins have been investigated as potential vaccine candidates. The structural proteins of the pneumococcal pilus type 1 are among the most recent ones to be investigated (13–16). The type 1 pilus was shown to act as an adhesin (17, 18), enhance colonization in a mouse model (19), and facilitate the formation of microcolonies and biofilms (20). Piliated pneumococcal strains induce significantly more TNF- α in a mouse model of intraperitoneal sepsis than pilus-negative isogenic controls (19).

At the same time, the role of the pneumococcal type 1 pilus in pneumococcal pathogenesis in humans remains controversial. We have previously demonstrated that the frequency of pilus genes was similar in strains isolated from the nasopharynx and blood cultures from infected children, putting in question the role of the pilus as a virulence factor (21). After the introduction of the pneumococcal conjugate vaccine in 2000 in the United States, the frequency of strains carrying the pilus genes declined dramatically, as a result of the association between the type 1 pilus and the capsular serotypes covered by the vaccine (21). However, several years later, replacement strains not covered by the vaccine became much more common, and the frequency of strains carrying pilus type 1 genes returned to pre-2000 levels, suggesting instead that the presence of these genes may confer an advantage to the organism (22). It is also striking that the prevalence of the pilus across different geographic areas and various pneumococcal clones is similar (23, 24). Complicating the picture further, our group and others recently showed that pilus expression is bistable among a clonal population (25, 26), such that only roughly 30% of cells derived from one clone express the pilus at any one time. This regulation appears to be negatively regulated by RrgA (25) and positively regulated by the RlrA-positive feedback loop (27).

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This intricate regulatory system suggests a complex combination of fitness benefits and costs of expression of the pilus to the bacterium. To elucidate this further, a better understanding of the interaction between the pilus and the host is warranted. In the studies described below, we examined the pro-inflamma-

tory and adherent properties of the type 1 pilus. In particular, we wished to evaluate whether the pilus engages the innate immune receptor TLR2 and to identify components of the pilus that may be responsible for the enhanced inflammation and binding observed with pilated strains. Here, we show that the structural ancillary pilus protein RrgA is a TLR2 agonist, most potently so when presented in an oligomerized form. We further demonstrate that a 49-amino acid region (P3) of the domain III (DomIII)³ of RrgA is necessary and sufficient for this activation. A pneumococcal mutant, in which P3 is deleted, is highly attenuated with respect to TLR2 activation despite no demonstrable defect in binding; similarly, this mutant is defective in the induction of TNF- α in mice following intraperitoneal injection, whereas no differences between the wild type strain and the mutant was apparent when TLR2^{-/-} mice were infected.

TABLE 1
Bacterial strains and plasmids used in this study

	Description	Source
Strain		
T4	Spontaneous Sm ^R derivative of Tigr4	On line
T4 Δ (pilus)	$\Delta rlrA$ - <i>srtD</i> :: <i>cm</i> Sm ^R , Cm ^R	25
T4 Δ rrgA	$\Delta rrgA$:: <i>Janus</i> Kan ^R	25
T4 Δ ABC	$\Delta rrgA$ - <i>rrgC</i> :: Sm ^R	This study
T4(<i>rrgA-II</i>)	$\Delta rrgA$:: <i>rrgA-II</i>	This study
T4*	Equivalent to T4 but transformed twice	This study
T4 Δ p3	$\Delta rrgAP3$	This study
Plasmids		
pAC1000	Derivative of pEV3	51
pQE30-rrgA	Wild type <i>rrgA</i>	25
pQE-30-rrgB	Wild type <i>rrgB</i>	25
pQE-30-rrgC	Wild type <i>rrgC</i>	This study
pET15b-DomIII	Domain III of <i>rrgA</i>	This study
pET15b-DomIII-Ply	Domain III of <i>rrgA</i> fused with <i>ply</i>	This study
pET15b-DomIII-Pdt	Domain III of <i>rrgA</i> fused with <i>pdt</i>	This study
pET15b-P1-Ply	Part 1 of Domain III of <i>rrgA</i>	This study
pET15b-P2-Ply	Part 2 of Domain III of <i>rrgA</i>	This study
pET15b-P3-Ply	Part 3 of Domain III of <i>rrgA</i>	This study

TABLE 2
Primers used in this study

Primers	Sequence	Source
<u>Cloning rrgC into pQE-30 plasmid</u>		
pQE30C5	5-ATCAGGATCCGCTCTGTGTTTTCTCTGTATGG-3	This study
pQE30C3	5-ATCCGCATGCATCAATCCGTGGTCGCTTGTTATTTTA-3	This study
<u>Cloning of Domain III</u>		
vW-RGD1- pET15b	5-TAACCTCGAGGGAGGAGGTGTTCTGGGAAAAACAGTGTATGAACAAAAAGAT-3	This study
vW-RGD2- pET15b	5-TTAAGGATCTGATTCTTTTCAGTTACGATGGTG-3	This study
<u>Cloning of Domain III-Ply or DomIII-Pdt</u>		
DIIPly1	5-GGTACATATGGGAAAAACAGTGTATGAACAA-3	This study
DIIPly22	5-CCCCGGATCCCGACCCCTCCGCCACCTGATTCTTTTCAGTTACGATGGTG-3	This study
DIIPly32	5-GTCGGGATCCGGGGAGGCGGTTCCGCAATAAAGCAGTAAATGACTTTA-3	This study
DIIPly4	5-CTCAGCTCAGCCTAGTCATTTTCTACCTTATC-3	This study
<u>Cloning of P1-Ply</u>		
Part1a	5-GTGCCGCGCGGCAGCCATATGTTGTCCATCATATCAAAAAT-3	This study
Part1b	5-GGAACCGCTCCCGGGATCCCGACCCCTCCGCCACCTTCTCCACTAGTTGCTTGCCT-3	This study
<u>Cloning of P2-Ply</u>		
Part2a	5-GTGCCGCGCGGCAGCCATATGGGAATACTATTAAGTGATTTT-3	This study
Part2b	5-GGAACCGCTCCCGGGATCCCGACCCCTCCGCCACCTCATAAAGTCTTATCTGT-3	This study
<u>Cloning of P3-Ply</u>		
Part3a	5-GTGCCGCGCGGCAGCCATATGAAAGGTGCTCCTGCAGCTTTC-3	This study
Part3b	5-GGAACCGCTCCCGGGATCCCGACCCCTCCGCCACCAAGAAAT-3	This study
<u>Deletion of rrgA-rrgC</u>		
DrrgA1	5-ACTCTTTGGACTCAGGGAAC-3	25
DrrgA23	5-TTCTCTGGAATAGGCATAGAAGATCTTTAAAAACAACCTCCATCACAA-3	This study
DrrgA33	5-TTGATGATGGAAGTTGTTTAAAGATCTTCTATGCCTATCCAGAGGAA-3	This study
DrrgC4n	5-ATGATGTACATTGAATATCACTAGGGCCCTTCCCTTATGCTTTTGGAC-3	This study
DrrgC5n	5-ATGATGTACATTGAATATCACTAGGGCCCTTCCCTTATGCTTTTGGAC-3	This study
DrrgC6	5-CAAACCTGTATGTGCCGAAT-3	This study
DABC1	5-TGCTTAAACAGGAGACACACATGTAGTATTCATATGTACATCAT-3	This study
DABC2	5-GTCCAAAAGCATAAGGAAAGGGCCCTAGTATTCATATGTACATCAT-3	This study
<u>Replacement of rrgA by rrgA-II</u>		
DrrgA1	5-ACTCTTTGGACTCAGGGAAC-3	25
rrgAcladelI2	5-CATGTGTCTCCCTGTTAAGCATTATTCATAGAACAAGTTAATCC-3	This study
rrgAcladelI3	5-GGATTAACCTGTTCTATGAATAATGCTTAAACAGGGAGACACATG-3	This study
rrgAcladelI4	5-CATAGATATTATCATTTTACACTTTACGGATGTTCCGTGTATAATAG-3	This study
rrgAcladelI5	5-CTATTATACACAGGAAACATCCGTAAGGTGATAGAAATGATAATATCTATG-3	This study
DrrgA6	5-CTGGATTGCTTTACCTTTGA-3	25
<u>DP3 deletion</u>		
DrrgA1	5-ACTCTTTGGACTCAGGGAAC-3	25
DP31	5-AGTATTAGAGCCCTCCACCTTCATAAAGTCTTATCTGT-3	This study
DP32	5-TATGAAGGTGGAGGGGCTCTAATACTGCTAAAATTACCAAT-3	This study
DrrgB4	5-GACCATCCACCTTCATAAAGTC-3	This study

EXPERIMENTAL PROCEDURES

Bacterial Strains and Reagents—Bacterial strains and plasmids are listed in Table 1. Primers used in this study are listed in Table 2. Pneumococci were grown in Todd Hewitt broth sup-

³ The abbreviations used are: DomIII, domain III; Ply, pneumolysin.

plemented with 0.5% yeast extract (THY) or on tryptic-soy agar with 5% sheep blood (TSA) plates. Antibiotics (300 $\mu\text{g}/\text{ml}$ kanamycin and 600 $\mu\text{g}/\text{ml}$ streptomycin) were added as needed. All pneumococcal mutants were constructed from a spontaneous streptomycin-resistant (Sm^R) strain (T4) derived from the serotype 4 clinical isolate TIGR4. Our T4* strain of reference is a strain in which the Janus cassette replacing the *rrgA* gene has been replaced *de novo* by the *rrgA* gene, to control for any effects of transformation. In particular, this strain was confirmed by flow cytometry to express the pilus in a bistable way, in a similar pattern as the original T4 strain (data not shown) (25). Killed pneumococcal strains were prepared by growth to $A_{600} = 0.5$ and then heat-inactivated at 58 °C for 1 h. *Escherichia coli* strains were grown in LB medium supplemented with 100 $\mu\text{g}/\text{ml}$ ampicillin as needed. Antibodies to RrgA and RrgB were obtained as described previously following purification of recombinant His₆-tagged RrgA and RrgB and immunization of guinea pigs and rabbits, respectively (at Cocalico Biologicals, Inc.) (25).

Human TNF- α was obtained from Human PeproTech (Rocky Hill, NJ). Ultrapure LPS and CLI-095, a cyclohexene derivative that specifically suppresses TLR4 signaling (28), were obtained from InvivoGen (San Diego).

Cloning Strategies—DNA cloning strategies were based on overlapping PCR and other previously described techniques (25). Pneumococcal mutants were generated using the bicistronic Janus cassette (29) and then sequenced. The strain T4 Δ (Pilus) was described previously (25). A strain deleted for the *rrgA* or *rrgB* using Janus was thus constructed. The cassette was then replaced by overlapping PCR strategy to create mutants of the RrgA protein. In particular, to evaluate the ability of RrgA of different clades, we replaced the endogenous *rrgA* gene in TIGR4 by the *rrgA-II* (clade II) gene using genomic DNA from a clinical pneumococcal isolate that expresses a clade II pilus type 1 (a gift of J. Finkelstein, Boston Children's Hospital). We also created a mutant with a deletion of 49 amino acids of the DomIII (Δ P3) of the RrgA protein. The DNA sequence of this mutant was confirmed. A T4 Δ ABC strain was generated by first replacing *rrgA*, *rrgB*, and *rrgC* genes by the Janus cassette, and then removing the Janus cassette by overlapping PCR. All constructs were confirmed by PCR and Western blotting analysis where applicable and sequence.

Cell Lines—Human embryonic kidney (HEK) cell lines stably transfected with TLR2 or TLR4 have been described previously (30). These cells were maintained in DMEM with 10% FBS, 10 $\mu\text{g}/\text{ml}$ ciprofloxacin, and 1 mg/ml G418. Murine macrophage cell lines derived from C57BL/6 mice that do or do not express TLR2 have also been described previously (31) and were maintained in DMEM with 10% FBS and 10 $\mu\text{g}/\text{ml}$ ciprofloxacin.

Protein Cloning, Purification, and Expression—His₆-tagged RrgA and RrgB were described previously (25). His₆-tagged RrgC was cloned into pQE30, whereas DomIII(Gly²¹²-Ser⁵⁹²), DomIII-Ply, DomIII-PdT, P1(Phe⁴⁰³-Glu⁴³⁷)-Ply, P2(Gly⁴²³-Glu⁴⁵⁸)-Ply and P3(Lys⁴⁵⁹-Ser⁵⁰⁷)-Ply were cloned into pET15b. Protein expression and purification were done as described previously (25). To separate the proteins by size, the

proteins obtained by elution from the nickel-nitrilotriacetic acid column were then passed over a gel filtration column (Superdex 200 column) in a buffer containing 20 mM Tris, pH 8.0, 150 mM NaCl as the running solution. The fractions containing target proteins of different sizes (reflecting different degrees of aggregation) were collected separately, tested by SDS-PAGE, and tested for TLR2 activity on HEK-TLR2 cells as described below. The protein concentrations were determined using a BCA protein assay kit from Bio-Rad.

Measurement of Inflammatory Responses—Enzyme-linked immunosorbent assays (ELISAs) for the determination of CXCL8/interleukin-8 (IL-8) were performed on supernatants from stimulated HEK-TLR2 or HEK-TLR4 cells. Twenty four-well ELISA plates were coated overnight with 6×10^5 HEK-TLR2 or HEK-TLR4 cells. Defined concentrations of either heat-killed bacteria or His tag-purified proteins were added to the wells for 16 h. Supernatants were then collected and analyzed for production of the human IL-8 using the DuoSet[®] ELISA kit following the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Similar experiments were performed using murine macrophage cell lines. In these experiments, 24-well ELISA plates were seeded with cells pretreated with CLI-095, an inhibitor of TLR4 responses, before the addition of defined concentrations of purified proteins for 16 h. Cell supernatants were harvested and assayed for murine TNF- α using the DuoSet ELISA kit (R&D Systems).

Bacterial Adherence Assays—Bacterial adherence assays were performed as described previously (40) with slight modifications. A549 human lung cells were obtained from the American Type Culture Collection (Manassas, VA) and grown according to recommended guidelines. Cells were seeded onto 24-well tissue culture plates to reach near-confluence at the time of the experiment. Bacterial cells were grown to mid-log exponential phase ($A_{600} = 0.5$) and then washed in PBS, and aliquots were saved in 20% glycerol at -80 °C. Individual aliquots were used for each experiment. About 10^7 cfu/ml were added to A549 cells, resulting in a multiplicity of infection of 10. After 3 h of incubation at 37 °C and 5% CO₂, cells were rinsed three times with PBS to remove nonadherent bacteria. A549 cells were then detached from the plate with 200 μl of 0.25% trypsin, 1 mM EDTA and solubilized with 800 μl of ice-cold 0.025% Triton X-100. Dilutions were plated on TSA plates. Percentage of binding was calculated by dividing the recovered number of bacteria by the input number after setting the T4 percentage at 100%. Each experiment was performed in triplicate, and the experiment was repeated three times on separate days. Representative results are shown in all cases.

Protein Adherence Assays—Immulon 2 HB 96-microwell plates (Thermo Scientific, Waltham, MA) were coated overnight at room temperature with 5 $\mu\text{g}/\text{ml}$ selected proteins. The next day, wells were washed and blocked with a solution of 1% bovine serum albumin (BSA) in PBS for 1 h. Wells were rinsed three times with PBS. HEK293, HEK-TLR2, or HEK-TLR4 cells, previously detached from their flask and resuspended in adequate cell media at a concentration of 5×10^5 cells/ml for 30 min at 37 °C, were then added onto the blocked plate for 1 h at 37 °C. Plates were then shaken gently to lift nonadherent cells

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and fixed for 15 min in 70% ethanol. After three PBS washes, cells were stained in crystal violet for 2 min and then rinsed thoroughly with water. Finally, the stain was recovered by cell solubilization in 10% acetic acid solution, and A_{590} was read. Each condition was performed in triplicate, and the experiment was repeated at least three times. Representative results are shown.

Flow Cytometry—Flow cytometry analyses to evaluate the percentage of cells that express the pilus were performed as described previously (25). Briefly, analysis was performed on a Beckman Coulter MoFlo Legacy flow cytometer. Post-acquisition analysis was performed using Beckman Coulter Summit 4.3 software and Treestar FlowJo 8.7.3. Heat-killed bacteria were blocked for 1 h in Buffer 0 (PBS 1× with 1% BSA), treated for 1 h with primary antibody in Buffer 1 (PBS 1×, 0.05% Tween 20, 1% BSA), washed once with Buffer 1, and then treated for 1 h with secondary antibody in Buffer 1. Bacteria were washed three times in Buffer 1 and analyzed by flow cytometry using MoFlo. The experiments were performed at room temperature. Alexa Fluor® 488 or 660 secondary antibodies from Invitrogen were used at a dilution of 1:50.

Mouse Sepsis Model—Four- to 6-week-old C57BL/6J female mice or TLR2^{-/-} isogenic controls were intraperitoneally injected with 200 μ l of THY containing 10⁸ cfu/ml of the appropriate pneumococcal strain; per experiment, 15 mice were injected with each pneumococcal strain. At 6 h post-infection, blood was taken on each mouse to measure the density of bacteremia and TNF- α concentration in serum samples. The density of bacteremia was evaluated by serial dilution of blood samples in PBS and plating on blood agar plates. TNF- α concentrations were measured using the mouse TNF- α /TNFSF1A DuoSet® ELISA kit following the manufacturer's conditions (R&D Systems). For each mouse, we report the TNF- α production at 6 h post-infection.

Statistical Analysis—TNF- α concentration and density of bacteremia in mice were compared by the Mann-Whitney *U* test, using PRISM (version 4.0a, GraphPad Software, Inc).

RESULTS

Pneumococcal Pilus Enhances TLR2-mediated Inflammatory Responses to Whole Killed Pneumococci—Previous work showed that deletion of the *rlrA* pathogenicity islet encoding the type 1 pilus of T4 leads to attenuated TNF- α and IL-6 responses in mice after intraperitoneal challenge (10). We began our investigations by evaluating whether the presence of the pilus on live or dead pneumococci had a different effect on cellular inflammatory responses. To this end, live or heat-killed T4 strain or a T4 strain with a deletion of the whole islet responsible for pilus expression (T4 Δ (*Pilus*)) were used to stimulate murine macrophage cell lines or human embryonic kidney cells (HEK293, expressing TLR2 or TLR4).

Pneumococci induce a range of inflammatory responses from cells due, at least in part, to TLR2, TLR4, and TLR9 responses to lipoteichoic acid (32), pneumolysin (33), and unmethylated CpG motifs in DNA (34), respectively, so that a specific effect of the pilus proteins may be difficult to observe. Therefore, we hypothesized that a differential effect of the pilus may best be initially observed in cells with a more limited rep-

ertoire of responses. *S. pneumoniae* is known to activate both TLR2 and TLR4 (32, 33, 35); thus, we wished to evaluate if either of these receptors may confer inflammatory responses to the pilus. Transfected HEK cells expressing either TLR2 or TLR4 were stimulated with heat-killed T4 strain or the T4 Δ (*Pilus*) strain. The concentration of IL-8 expression in cell supernatants, a marker of TLR2 or TLR4 activation in HEK-TLR2 or HEK-TLR4 cells, respectively, was determined after 16 h of incubation by ELISA (Fig. 1A). As expected, stimulation of HEK-TLR2 cells with killed wild type pneumococci elicited IL-8 expression in a dose-dependent manner (Fig. 1A); in comparison, IL-8 expression was significantly attenuated when HEK-TLR2 cells were stimulated with the T4 Δ (*Pilus*), suggesting that the pneumococcal pilus augments the TLR2-dependent responses to killed pneumococci. Identical results were obtained with a strain carrying a deletion of the *rrgA*, *rrgB*, and *rrgC* genes or a strain with a deletion of the pilus regulator *rlrA* (data not shown) suggesting that this decrease in TLR2 activation is likely due to the absence of these structural proteins rather than the absence of the RlrA transcription factor.

RrgA Protein Activates TLR2—The findings above could be consistent with two nonmutually exclusive hypotheses as follows: (a) one or more proteins that form the pilus (RrgA, RrgB, and RrgC proteins) may be TLR2 agonists, and/or (b) because the pilus has been shown to enhance binding of pneumococci to epithelial cells via the RrgA protein, the increased inflammatory response may be a direct result of increased binding of pilated bacteria to epithelial cells. To evaluate these possibilities, we purified the three structural proteins of the pilus. Consistent with previous findings (17), we confirmed that the recombinant RrgA immobilized to a surface functions as an adhesin, to a similar extent on HEK293, HEK-TLR2, or HEK-TLR4 cells (Fig. 1B); in contrast, we were unable to demonstrate such property for the RrgB or RrgC protein. To test whether any of these proteins activates TLR2, we stimulated HEK-TLR2 (Fig. 1C) and control HEK-TLR4 cells with a range of concentrations of proteins (Fig. 1D). No IL-8 expression was detected from the HEK-TLR4 cells with any of the single proteins confirming the data obtained when the cells were stimulated with whole bacteria (Fig. 1D) (these cells do not express MD-2 and are thus largely unresponsive to any possible contaminating LPS). In contrast, activation of HEK-TLR2 cells was readily observed with RrgA, with RrgB and RrgC being far less potent (Fig. 1C). Thus, we decided to focus the remainder of our studies on RrgA.

Because most previously described TLR2 ligands or agonists are either lipids or lipid-modified polysaccharides or proteins, we asked if the TLR2 activity of RrgA is due to intrinsic lipidation of the molecule. Bioinformatic analysis on the sequence of RrgA did not identify any obvious lipidation motif or site. Furthermore, a MALDI-TOF mass spectrometry analysis was performed on the purified full-length RrgA protein (PrimmBiotech, Cambridge, MA), and no lipidated peptide fragment could be identified. These results strongly suggest that the TLR2 activity of RrgA is mediated by the protein itself rather than by the presence of a lipid moiety in the molecule.

To obtain further confirmation that RrgA is a TLR2 activator, we repeated similar experiments in HEK-TLR2 cells using

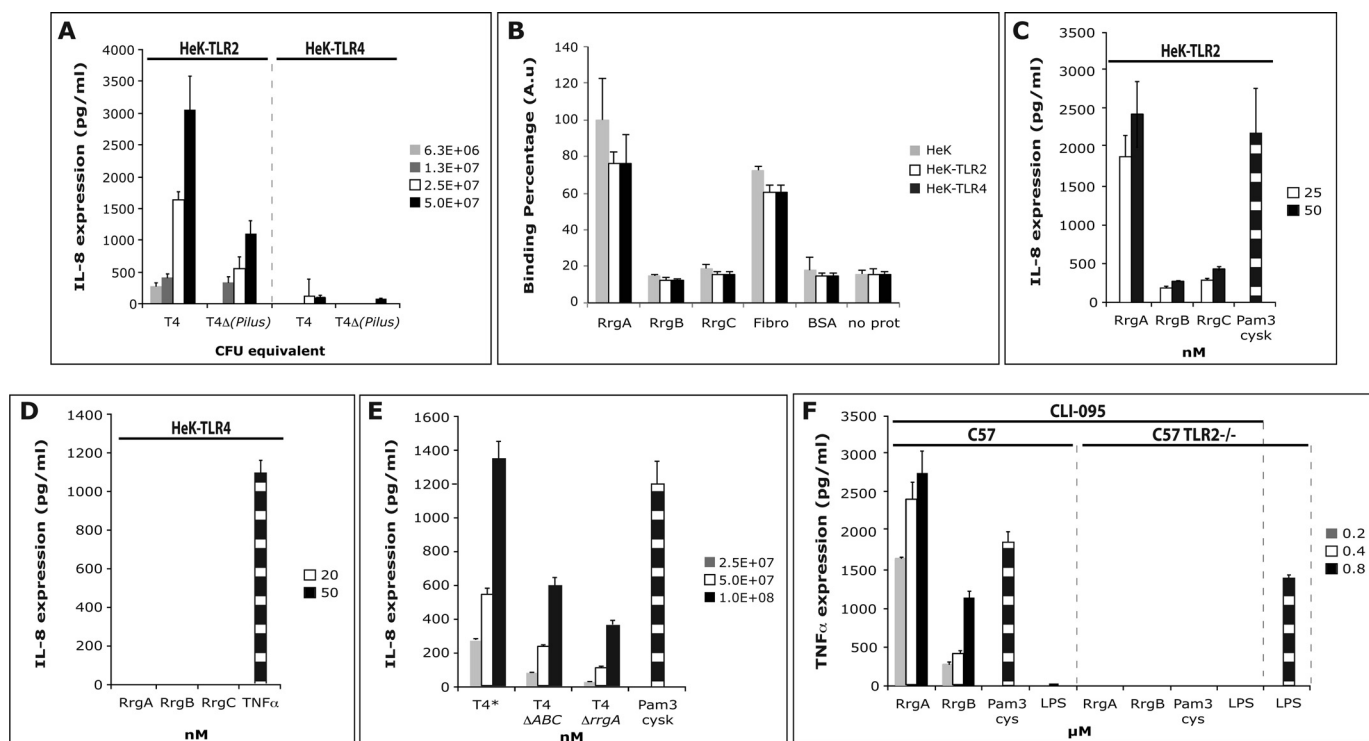


FIGURE 1. Type 1 pneumococcal pilus enhances TLR2-dependent inflammatory responses in human epithelial cells. *A*, transfected HEK cells expressing TLR2 or TLR4 were stimulated with increasing concentrations of heat-killed T4 or T4Δ(*Pilus*) pneumococcal strains. IL-8 expression was quantified by ELISA after 16 h of incubation. Although heat-killed pneumococci did not stimulate IL-8 responses in HEK-TLR4 cells, a dose-dependent IL-8 elicitation in HEK-TLR2 cells was observed and was significantly more pronounced when pilated bacteria were used. *B*, 96-well plates were coated with 5 μg/ml of various proteins (bovine serum albumin (BSA), fibronectin, RrgA, RrgB, and RrgC or without protein coating) overnight at room temperature. HEK293, HEK-TLR2, or HEK-TLR4 cells were added to the wells at a concentration of 5×10^5 cells/ml for 1 h at 37 °C. Wells were washed and bound cells were stained with crystal violet; A_{590} measured. Although no binding could be demonstrated with RrgB or RrgC, RrgA demonstrated strong binding to cells. *C* and *D*, increasing concentrations of purified proteins RrgA, RrgB, and RrgC were used as stimuli for HEK-TLR2 (*C*) and HEK-TLR4 (*D*) cells; supernatants were assayed for IL-8 expression after 16 h of incubation. IL-8 expression in HEK-TLR4 cells was negligible, whereas the pilus proteins induced dose-dependent expression of IL-8 from HEK-TLR2 cells. On a molar basis, the RrgA protein was the most potent activator of TLR2. The TLR2 agonist Pam₃Cysk (1 μg/ml) was used as a control in HEK-TLR2 cells; for HEK-TLR4 cells, which do not express MD-2, recombinant TNF-α (50 ng/ml) was used. *E*, IL-8 responses of HEK-TLR2 cells following stimulation with heat-killed pneumococcal preparations (wild type T4* and strains T4ΔABC and T4ΔrrgA) at the listed concentrations (per ml) for 18 h. Cell supernatants were then collected and assayed for IL-8 concentration. There was a clear dose-dependent IL-8 response, greater in the wild type bacterial preparation when compared with the two other strains, which both stimulated the cells to a similar degree, implying a role of RrgA in TLR2-dependent responses in the context of whole bacteria. *F*, TLR2-dependent responses to pilus proteins evaluated in murine macrophages. Purified proteins RrgA, RrgB, and RrgC were used to stimulate murine macrophages derived from C57BL/6 and isogenic TLR2^{-/-} knock-out mice. To eliminate confounding by possibly LPS contamination, the stimuli were all pretreated with the specific TLR4 inhibitor CLI-095, as described in the text. Both RrgA and RrgB elicited dose- and TLR2-dependent responses from the cells. Controls included Pam₃Cysk (1 μg/ml) and LPS (with and without CLI-095 pretreatment, 100 ng/ml). All these experiments were performed at least three times, with triplicate wells per experiment. Representative results are shown, and error bars represent standard deviations.

killed preparations of pneumococci, including a strain that lacks either the three structural proteins or RrgA. As we have shown previously (25), the T4ΔrrgA strain makes a polymerized pilus (as detected by Western blotting analysis using RrgB antibodies). As shown in Fig. 1*E*, in the absence of RrgA, the killed pneumococcal preparation is far less stimulatory of HEK-TLR2 cells, to the same extent as the T4ΔABC strain. These data therefore imply that, within the context of the intact pneumococcal cell, RrgA is the main activator of TLR2.

Recognizing that HEK-TLR2 cells represent a somewhat artificial construct, we next evaluated the TLR2 stimulatory properties of RrgA and RrgB in murine macrophages without or with a deletion of TLR2. Because these proteins were purified in *E. coli*, and to remove the effect of any potential LPS contamination, all the stimuli in these experiments were pretreated with CLI-095, a specific inhibitor of TLR4 signaling (28). As shown in Fig. 1*F*, RrgA and, to a lesser extent, RrgB stimulated TNF-α release from the wild type macrophages, although this

response was completely abrogated in TLR2^{-/-} macrophages, implying that this response, now observed in murine macrophages, is indeed TLR2-dependent.

Aggregation Enhances TLR2 Activity of RrgA—By gel filtration chromatography, the purified RrgA protein was separated by size into three major fractions, representing different aggregation states. By the apparent molecular weight calculated from the chromatography profile, we called these three fractions monomer (M1), small aggregates (SA1), and large aggregates (LA1), respectively (Fig. 2, *inset*). The three fractions, along with the nonfractionated RrgA protein, were used to stimulate HEK-TLR2 cells at equal concentrations. Strikingly, whereas the monomeric RrgA (M1) fraction led to very little TLR2 activation, stimulation of HEK-TLR2 cells with the same amount of protein in the SA1 or LA1 fractions gave much more potent TLR2 activation, by inducing a 3.6-fold (SA1) and 21-fold (LA1) higher concentration of released IL-8 than did the monomer RrgA (Fig. 2). This result suggests that aggregation of RrgA significantly enhances TLR2 activation.

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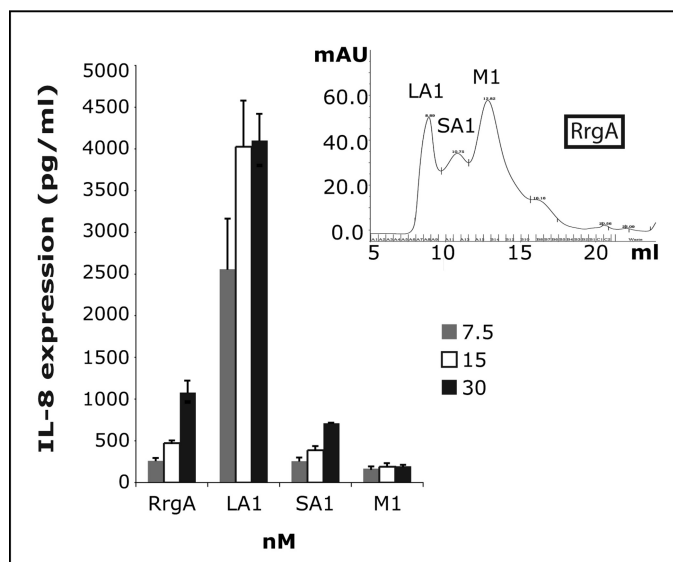


FIGURE 2. TLR2 preferentially recognizes large aggregates of the RrgA protein. *Inset*, RrgA protein was further purified on a Sepharose column (*inset*). Different sizes of the protein were observed, reflecting a monomeric form (M1), a small aggregate (SA1), and large aggregate (LA1). Stimulation of HEK-TLR2 cells with equimolar amounts of RrgA proteins of different size is shown in the main figure. The larger aggregates are significantly more stimulatory of TLR2 than the smaller aggregates. *Error bars* indicate standard deviations. *MAU*, milli-absorbance units.

Domain III of the RrgA Protein Activates TLR2—Crystal structure analysis of the RrgA protein reveals four major domains (Fig. 4A) (36), where domain IV is thought to be responsible for the interaction with RrgB, and domain III is extended to the outside of the pilus. We hypothesized that domain III may be more likely to interact with cell surface receptors, such as TLR2. To evaluate this possibility, we expressed and purified domain III of RrgA. By gel filtration chromatography, the recombinant domain III was found to form a smaller rather than a larger aggregate as the full-length RrgA (Fig. 3A, *inset*). When used to stimulate HEK-TLR2 cells, the purified domain III did not induce a significant IL-8 response (Fig. 3A). To further oligomerize domain III, a fusion protein was made by genetically fusing domain III with pneumolysin (Ply), a toxin known to be able to lyse cells by forming a large pore-like oligomer on the cell membrane. A similar fusion protein was constructed in which domain III was fused to PdT, the pneumolysin mutant (W433F, D385N, and C428G) that is unable to oligomerize (37). As expected (38), the domain III-pneumolysin fusion protein (DomIII-Ply) was present in three different forms in solution as follows: a monomer (M2), a small oligomer (SO2), or a large oligomer (LO2) (Fig. 3B). In contrast, the domain III-PdT fusion protein (DomIII-PdT) was present only as a monomer in solution (Fig. 3B). When these fusion proteins were used to stimulate HEK-TLR2 cells, DomIII-Ply, but not DomIII-PdT, induced strong inflammatory responses comparable to a molar basis with the one observed with the RrgA protein (Fig. 3C). Furthermore, when stimulating cells with DomIII-Ply fractions of different sizes, the large oligomer (LO2) showed significantly higher TLR2 activity than the small oligomer (SO2) or the monomer (M2) (Fig. 3D), consistent with our prior observations with RrgA.

A 49-Amino Acid Region (P3) of the DomIII of the RrgA Protein Is Necessary and Sufficient to Activate TLR2—We truncated RrgA further to more precisely identify a region that may be responsible for TLR2 activation. Domain III of the RrgA protein can be divided into two parts as follows: a well structured core domain (Fig. 4A, *gray structure*), which is physically connected to domain II, and a peripheral fragment that is very much exposed at the distal end of RrgA and may be easily accessible with less steric hindrance (Fig. 4A). Thus, we tested if this peripheral fragment would activate TLR2 responses. Three peptides of 35 (P1, Fig. 4A, in *blue*), 36 (P2, Fig. 4A, in *green*), and 49 amino acids (P3, Fig. 4A, in *red*) in length were designed to cover the sequence from residue 403 to 507. The three peptides were fused to Ply and tested for TLR2 activation (Fig. 4B). When tested on HEK-TLR2 cells, only the P3-Ply fusion protein was able to activate TLR2 and at roughly the same potency as the RrgA protein, suggesting that this 49-amino acid sequence, when presented as an oligomer, is sufficient to activate TLR2 (Fig. 4B).

The type 1 pneumococcal pilus can be divided into clades I, II, and III (to which the TIGR4 type 1 pilus is most closely related) (39). We evaluated the sequence similarities between the three clades, which revealed that although the P3 region of clades I and III were highly conserved, there were greater differences between clade II and our RrgA protein (Fig. 4C). There is >60% homology at the amino acid level in the P3 region, with the absence of five residues in RrgA II (corresponding to the last β -sheet motif in RrgA) just before a very conserved C-terminal loop region (Trp⁴⁹⁶–Ser⁵⁰⁷). To evaluate whether the clade II RrgA protein can activate TLR2, we replaced the *rrgA* gene found in our TIGR4 strain with the clade II gene, as described under “Experimental Procedures.” This new strain (designated T4(*rrgA II*)) was compared with the T4 strain for pilus expression, formation, and its ability to stimulate HEK-TLR2 cells. As shown, there were no appreciable differences in the amount of pilus made by the two strains nor the appearance of the pilus by Western blotting (Fig. 4D). Furthermore, there were no differences in the ability of either killed bacterial preparations to activate TLR2, and both were significantly more active when compared with a strain with a deletion of *rrgABC* (Fig. 4E). Thus, we conclude that RrgA from clade II can similarly activate TLR2, perhaps due to the homology with clade III.

We and others showed that individual clones of pilated pneumococci can have very different pilus expression patterns, such that the comparison of a wild type strain with one carrying a modification in the *rrgA* gene should include an examination of the pattern of pilus expression (25, 26). To control for these issues, we confirmed by Western blot and flow cytometry studies that a pneumococcal mutant carrying the *rrgA* gene with a deletion of the corresponding sequence of the P3 (T4 Δ P3) expresses the pilus in the same pattern as the wild type strain (Fig. 4F). HEK-TLR2 cells were then stimulated with heat-killed preparations of the three pneumococcal strain as follows: T4*, T4 Δ (*pilus*), and T4 Δ P3. The T4 Δ P3 mutant stimulated HEK-TLR2 cells to the same extent as the T4 Δ (*pilus*) strain (Fig. 4G), thereby confirming the P3 region as necessary for RrgA-induced TLR2 activation.

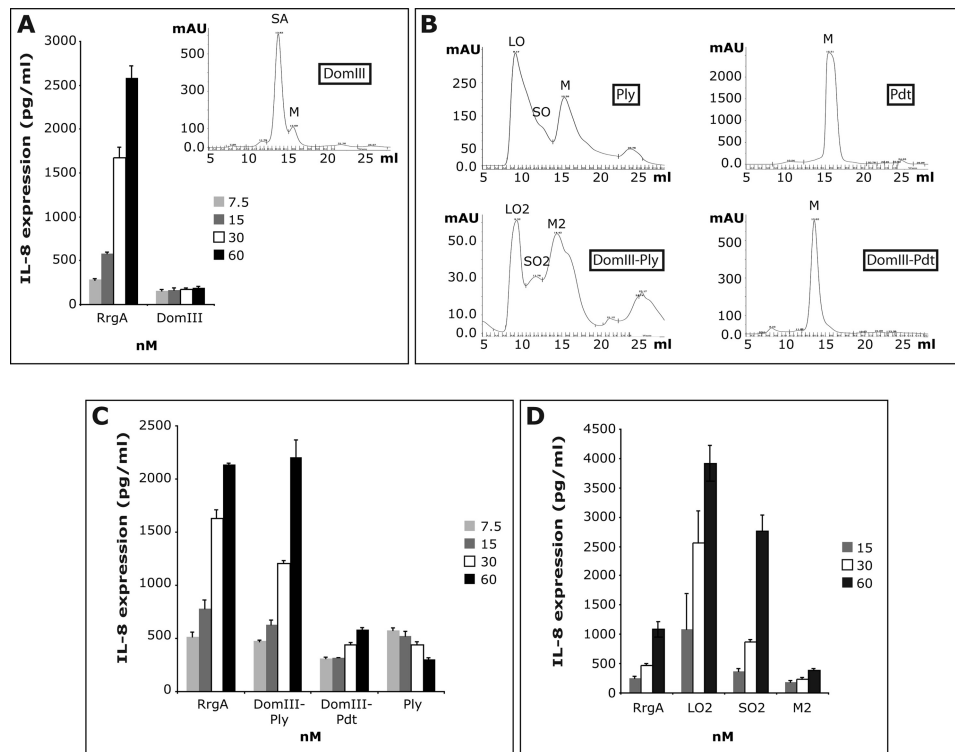


FIGURE 3. Oligomerized DomIII of the RrgA protein activates TLR2. *A*, monomeric form of DomIII does not activate TLR2. DomIII of the RrgA protein was expressed in *E. coli* and separated on a Sepharose column. DomIII is mainly found as monomer (*M*) or small aggregate (*SA*). Stimulation of HEK-TLR2 cells with DomIII does not result in IL-8 production. *B*, Ply, PdT (a nonhemolytic mutant of pneumolysin), DomIII-Ply, and DomIII-PdT were separated by size on a Sepharose column. Ply or a fusion of DomIII with Ply forms large oligomers, whereas PdT or the DomIII-PdT fusion only forms monomers. *mAU*, milli-absorbance units. *C*, oligomerized DomIII-Ply activates TLR2. Supernatants from HEK-TLR2 cells stimulated with RrgA, DomIII-Ply, DomIII-PdT, and Ply proteins were assayed for IL-8 production. The DomIII-Ply fusion protein is as potent as the RrgA protein on a molar basis with respect to TLR2 stimulation. *D*, larger oligomers of the DomIII-Ply fusion protein are more potent activators of TLR2. The fusion protein was separated by passage over a Sepharose column, revealing different stages of protein oligomerization (labeled *M2*, *SO2*, and *LO2*, for the monomeric, small oligomers, and large oligomers, respectively) of the fusion protein. The large oligomer of DomIII-Ply is the most potent activator of TLR2 as measured by IL-8 production in HEK-TLR2 cells. *Error bars* indicate standard deviations.

These data thus support the hypothesis that the enhanced inflammatory response to pilated pneumococci is the result of TLR2 activation by pneumococcal pilus proteins, most notably RrgA; indeed, when the P3 domain is removed, TLR2 responses were significantly reduced. The possibility remained, however, that this reduction in TLR2 activation might also be due to decreased binding of bacteria to cells rather than the elimination of the putative amino acid sequence responsible for TLR2 activation. As shown in Fig. 4*H*, however, the T4 Δ P3 mutant pneumococcal strain has similar binding capacity as the T4* strain in the adhesion assay. Thus, we conclude that the P3 region of DomIII of the RrgA protein is both necessary and sufficient for TLR2 activation and that this property is not due to altered binding.

P3 Region of Domain III of the RrgA Protein Contributes, in a TLR2-dependent Fashion, to the Density of Bacteremia and Pro-inflammatory Response to Live Pneumococci in an Intraperitoneal Infection Model—The virulence and inflammatory response to T4* and T4 Δ P3 strains were compared in a mouse model of intraperitoneal infection. Mice ($n = 15$ per group) were infected with 10^8 cfu of either T4* or the T4 Δ P3 mutant by intraperitoneal injection. At 6 h post-infection, mice were bled, and levels of bacteremia and TNF- α in the serum were determined. Both bacteremia and TNF- α levels were significantly higher in the mice that received T4* *versus* the T4 Δ P3 strains (comparison of density of bacteremia and TNF- α concentra-

tions, $p = 0.03$ and $p = 0.004$, respectively by Mann-Whitney U analysis). The density of bacteremia and TNF- α concentrations were highly correlated (Spearman $\rho = 0.59$, $p = 0.0006$). To confirm that these differences are in fact dependent on TLR2, a similar experiment was performed in C57BL/6 TLR2 $^{-/-}$ mice ($n = 5$ per group). The density of bacteremia in WT *versus* TLR2 $^{-/-}$ mice was not statistically different (Fig. 5*D*) nor was the concentration of TNF- α from serum obtained 6 h post-infection (Fig. 5*E*), with an excellent correlation between bacteremia and TNF- α concentration (Fig. 5*F*). We conclude that the P3 domain of the RrgA protein is a critical and TLR2-dependent determinant of the density of bacteremia and pro-inflammatory response to invasive disease due to pilated pneumococci.

DISCUSSION

Recognition of bacterial components by the innate immune response is an effective method to protect the host against various pathogens (40). In particular, the interaction of bacterial components with Toll-like receptors has been associated with protection against viral and bacterial diseases (33, 41–46). Since the first description of Toll-like receptors in humans, there have been numerous reports of various agonists for each receptor. TLR2 is particularly interesting in this respect, as it appears to recognize the broadest range of bacterial compounds of all the human Toll-like receptors studied to date. The

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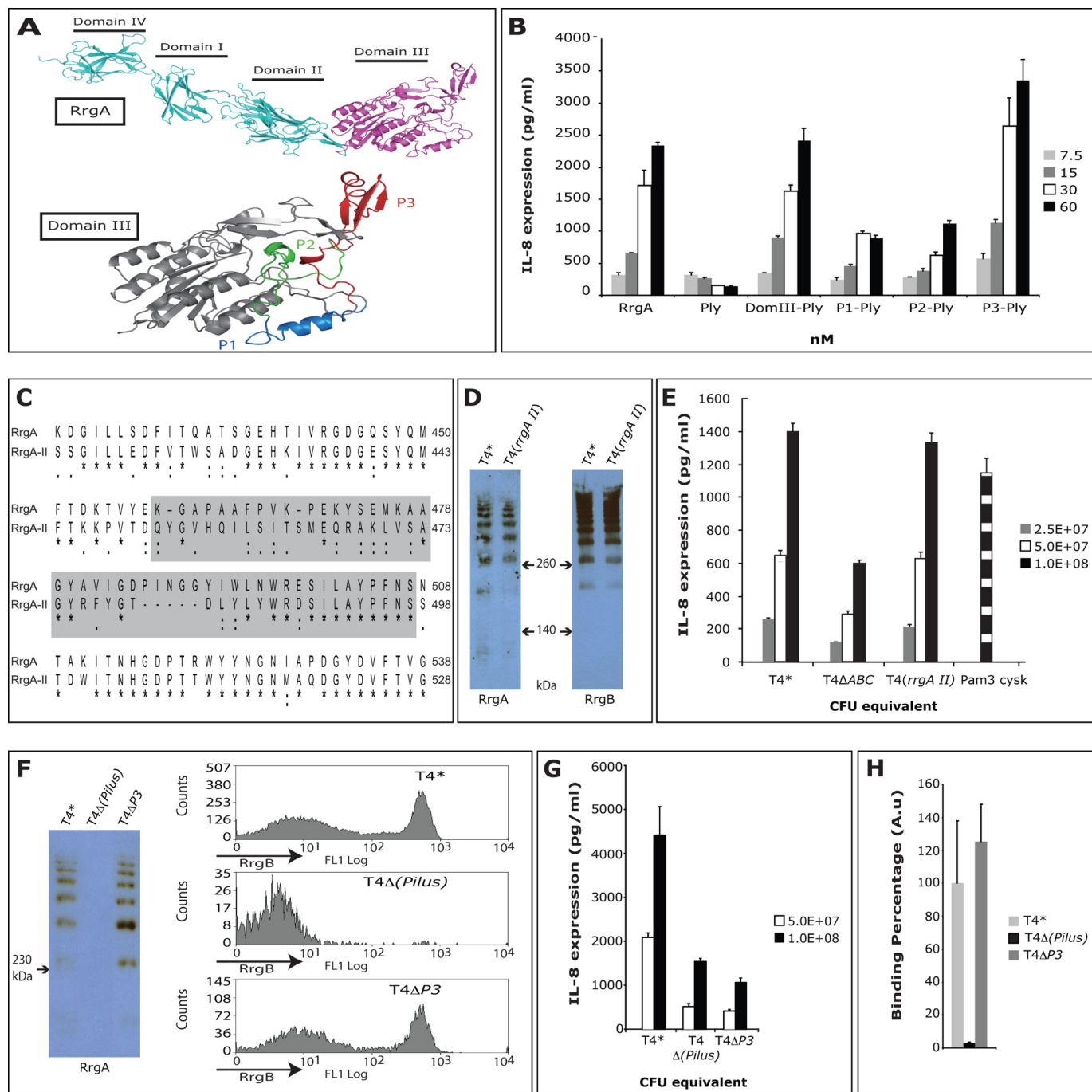


FIGURE 4. P3 region of DomIII is necessary and sufficient for pilus-dependent TLR2 activation. *A*, schematic arrangement and tertiary fold of RrgA (*upper panel*) (36). Representation of Domain III and the three defined peptides P1 (*blue*), P2 (*green*), and P3 (*red*) (*lower panel*). *B*, P3-Ply is the most potent of the three fusion proteins with respect to TLR2 activation, as determined by IL-8 production by HEK-TLR2 cells. *C*, sequence alignment of the P3 region of RrgA from T4* and RrgA clade II (designated RrgA-II). *D*, Western blotting analysis of the T4* and T4(*rrgA II*) strains probing for the RrgA and RrgB protein. The amount and laddering of the pili in both strains appear similar. *E*, stimulation of HEK-TLR2 cells with killed pneumococcal preparation of T4* and the clade II-expressing T4 (T4(*rrgA II*)) reveal similar abilities to elicit TLR2-dependent responses; a killed T4ΔABC strain was used as a comparator. *F*, T4ΔP3 mutant lacking the P3 region of the RrgA protein demonstrates no change in pilus synthesis or quantity on cell wall extract, as determined by Western blotting analysis. Additionally, the pattern of pilus expression is similar between the wild type T4* strain and this T4ΔP3 mutant by flow cytometry using an RrgA antibody. *G*, P3 is necessary for pilus-dependent TLR2 responses following stimulation with killed bacterial cells. The T4ΔP3 mutant was compared with a T4* or T4Δ(*Pilus*)-killed strain with respect to TLR2 activation in HEK-TLR2 cells. The pilus-dependent enhancement of TLR2 responses is abrogated in the T4ΔP3 mutant, which is indistinguishable from the T4Δ(*Pilus*) strain in terms of TLR2 activity, implying that the P3 region is necessary for this response to whole bacteria. *H*, T4ΔP3 mutant is not altered in binding capacity. A binding assay on A549 cells was performed. The T4ΔP3 and the T4* strains both bind to these cells similarly. *Error bars* indicate standard deviations.

broad range of activators of this receptor is exemplified by the growing list of identified molecules that interact with TLR2, which now includes lipopolysaccharides of certain bacteria,

lipoproteins, glycoproteins, zymosan, peptidoglycan, and lipoteichoic acids (reviewed in Ref. 47). Here, we add to this list of bacterial TLR2 agonists by demonstrating that the pneumococ-

C57BL/6 mice

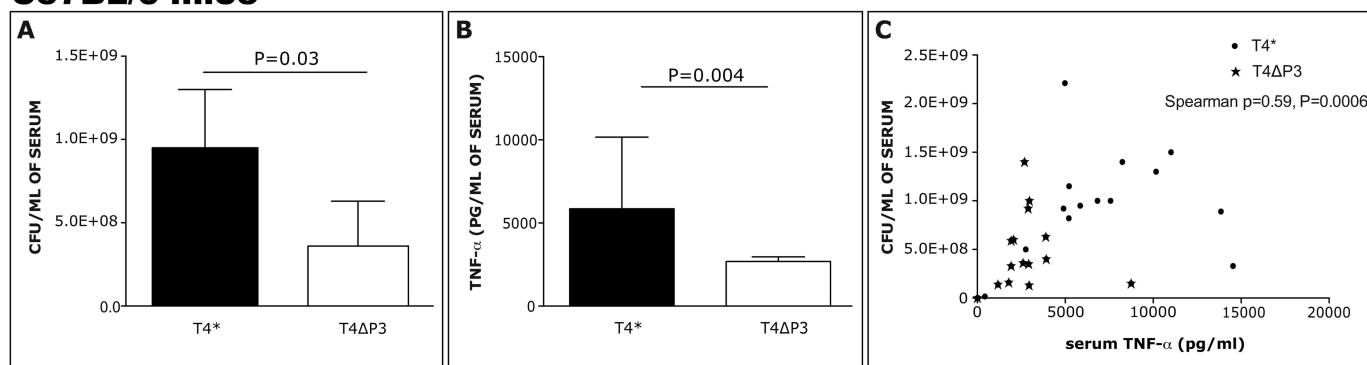
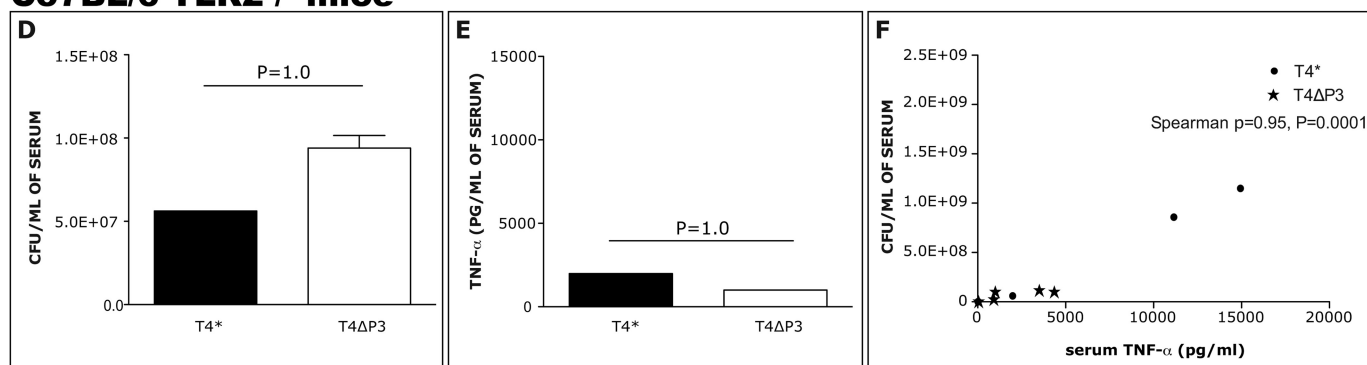
C57BL/6 TLR2^{-/-} mice

FIGURE 5. Density of bacteremia and the TLR2-dependent pro-inflammatory response in an *in vivo* mouse model are dependent on the P3 domain. Intraperitoneal infections were performed on mice with either live T4* or T4ΔP3 bacterial strains in wild type (A–C) or TLR2^{-/-} (D–F) C57BL/6 mice. Blood from each mouse was recovered at 6 h post-infection for quantification of bacteremia and TNF-α response. The bars in the figure represents the median and interquartile range of bacteremia (A and D) and TNF-α concentration in serum relative to the density of bacteremia in cfu (B and E) at 6 h post-infection. *p* values for comparisons of density of bacteremia and TNF-α were calculated by Mann-Whitney U analysis; statistical correlation between these two variables was examined using nonparametric methods (Spearman method) (C and F).

cal type 1 pilus is a determinant of the TLR2-dependent response to whole bacteria and is, in itself, a TLR2 agonist.

Published data by our group and others had previously identified pneumolysin and lipoteichoic acid as TLR4 and TLR2 agonists, respectively (32, 33, 48). Here, we used pneumolysin, which does not activate TLR2, as a tool for oligomerization. It had also been noted that, in general, the inflammatory response to *S. pneumoniae* is less pronounced than that of another Gram-positive bacterium, *Streptococcus agalactiae* (49), but these studies were based on pneumococcal strain D39, which is nonpiliated (19). The pilus has been shown to play an important role in the virulence and inflammatory response to pneumococcus (19); here, we show that this property is mediated, at least in part, by TLR2. Our data using whole bacteria as well as purified pilus components further implicate a specific protein, RrgA, as the major determinant of TLR2 activity. RrgA has been described as an adhesin and binds to fibronectin, collagen 1, and laminin (17, 50). Pilus- or RrgA-negative mutants of pneumococci have been shown to be relatively less virulent than their wild type isogenic controls. In the absence of the pilus, the TNF-α response is significantly reduced (19). RrgA-mutant pneumococci are defective in binding to epithelial cells and colonizing the upper respiratory tract of mice (17). Here, the molecular basis for these properties is examined, with the demonstration that the RrgA protein significantly induces a TLR2-dependent inflammatory response *in vitro*. We examined this

interaction in more detail, with the demonstration that P3 of domain III is both necessary and sufficient for pilus-dependent TLR2 activation, as well as the virulence (as measured by density of bacteremia) and induction of TNF-α responses in an invasive disease model in mice. We showed that this decrease in virulence is not due to a reduction of binding of the mutated RrgA protein, thus implying that the regions of the protein that contributes to inflammation are distinct from those mediating the adhesive properties of RrgA.

It is worth noting that the impact of the pilus on *in vitro* inflammatory responses could be demonstrated not only using HEK-TLR2 cells but also murine macrophages, which have a fuller complement of innate immune responses. Additionally, the role of the P3 domain could also be demonstrated *in vivo* in our intraperitoneal challenge model. This result is somewhat surprising, given the redundancy of the inflammatory response to whole bacteria, which contain stimuli for a number of receptors. Our experiments thus clearly point to the importance of the pilus (and in particular, the 49-amino acid region of domain III of RrgA) as a determinant of virulence and inflammatory responses in the context of whole bacteria.

Another unexpected finding of our work is that RrgA protein presented either as part of an oligomer or as an aggregate is far more potent as an activator of TLR2 than the monomeric form. The results of our evaluation of DomIII are consistent with this observation, as DomIII alone (which forms small aggregates) or

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DomIII-PdT did not activate HEK-TLR2 cells whereas the DomIII-Ply fusion protein was a potent TLR2 agonist. In this respect, our findings are reminiscent of the discovery that Dec-1, a human innate immune receptor, can similarly discriminate between large aggregates of β -glucans and smaller soluble forms (51). Such a discrimination by size or degree of aggregation may be important to allow the host immune response to react differentially to pathogens versus secreted or released antigens derived from pathogens. Furthermore, these results also suggest the possibility that by oligomerizing small peptides (such as P3), defined nonlipidated molecules with potent TLR2 activity may be created with a possible role as adjuvants for vaccines.

Many puzzling questions regarding the type 1 pneumococcal pilus remain. In particular, if the pilus increases adherence to host cells, why would this adhesion be present in only a minority of strains, and why would its expression, as we have shown (25), be restricted to a subpopulation of cells that contain the pathogenicity islet? Data presented here suggest at least one possible explanation, which deserves further study. Although the type 1 pilus may indeed increase the adherence of pneumococcal cells in the respiratory tract, recognition of this structure by a component of the innate immune system may limit the advantage that increased adherence to epithelial cells may otherwise provide. The recognition of the pilus by TLR2 may thus counterbalance the potential benefit of the pilus to the pathogen.

In conclusion, we show here that the RrgA protein of the type 1 pneumococcal pilus contributes to the recognition of the pilus by host TLR2. Peptide 3 of DomIII in particular is both necessary and sufficient for this TLR2-mediated inflammatory response, independently of any effect on cell adhesion. Studies are ongoing to examine the molecular basis for the interaction between RrgA and TLR2, the mechanism of P3 recognition by TLR2, and the possible implications with respect to the role of the pilus in pneumococcal fitness and virulence.

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