Bordetella pertussis Lipid A Recognition by Toll-like Receptor 4 and MD-2 Is Dependent on Distinct Charged and Uncharged Interfaces*

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Background: Glucosamine modification of Bordetella pertussis lipid A stimulates host-specific immunity.

Results: Charged amino acids mediate host-specific responses to glucosamine modification, whereas uncharged amino acids are critical for responses to B. pertussis lipid A.

Conclusion: Multiple sites of interaction, both conserved and unique, are utilized for discrimination of lipid A variants.

Significance: This is the first study to systematically dissect penta-acylated lipid A recognition.

Lipid A in LPS activates innate immunity through the Toll-like receptor 4 (TLR4)-MD-2 complex on host cells. Variation in lipid A has significant consequences for TLR4 activation and thus may be a means by which Gram-negative bacteria modulate host immunity. However, although even minor changes in lipid A structure have been shown to affect downstream immune responses, the mechanism by which the TLR4-MD-2 receptor complex recognizes these changes is not well understood. We previously showed that strain BP338 of the human pathogen Bordetella pertussis, the causative agent of whooping cough, modifies its lipid A by the addition of glucosamines moieties that promote TLR4 activation in human, but not mouse, macrophages. Using site-directed mutagenesis and an NFκB reporter assay screen, we have identified several charged amino acid residues in TLR4 and MD-2 that are important for these species-specific responses; some of these are novel for responses to penta-acyl B. pertussis LPS, and their mutation does not affect the response to hexa-acylated Escherichia coli LPS or tetra-acylated lipid IVa. We additionally show evidence that suggests that recognition of penta-acylated B. pertussis lipid A is dependent on uncharged amino acids in TLR4 and MD-2 and that this is true for both human and mouse TLR4-MD-2 receptors. Taken together, we have demonstrated that the TLR4-MD-2 receptor complex recognizes variation in lipid A molecules using multiple sites for receptor-ligand interaction and propose that host-specific immunity to a particular Gram-negative bacterium is, at least in part, mediated by very subtle tuning of one of the earliest interactions at the host-pathogen interface.

Lipopolysaccharide (LPS) is found on the surface of most Gram-negative bacteria and is recognized by the host innate immune system during infection by the TLR4-MD-2 receptor complex (1). Lipid A is the bioactive component of LPS and is sufficient to activate TLR4 (2). The best characterized lipid A structure is hexa-acylated lipid A from Escherichia coli, which consists of a di-glucosamine backbone, two negatively charged phosphate groups, and six acyl chains (3). This form of lipid A stimulates robust activation of the transcription factor nuclear factor κB (NFκB) through both human and mouse TLR4-MD-2 receptors. Crystal structure analysis shows a dimer of human or mouse TLR4-MD-2 receptors in complex with E. coli LPS (4, 5). This dimerization is thought to be necessary to initiate downstream signaling, including recruitment of the adaptors MyD88 and TRIF, activation of NFκB and IRF3, and the production of proinflammatory cytokines and type I interferons, respectively (6).

The mechanism by which E. coli lipid A is recognized by human and mouse TLR4-MD-2 is similar between human and mouse receptors; first, the acyl chains of lipid A are accommodated in a binding “pocket” structure of the co-receptor MD-2. From this position, the negatively charged phosphate groups can engage positively charged amino acid residues in TLR4. However, the phosphate groups on lipid A can also interact with positively charged amino acid residues in a second TLR4 molecule (TLR4*), which may help to facilitate dimerization of the two TLR4-MD-2 receptor complexes. Additionally, because one of the acyl chains on hexa-acylated E. coli lipid A sticks out of the pocket and lies on the surface of MD-2, this also promotes dimerization via interaction with uncharged amino acids in TLR4* (4, 5). Uncharged amino acids in TLR4* (Phe-440 and Phe-463 in human TLR4 and Phe-438, Phe-461 in mouse TLR4) have also been proposed to engage MD-2 and support receptor activation (5, 7, 8).

Variations in the structure of lipid A can affect its ability to activate NFκB (9), and several lipid A modifying enzymes have been described in Gram-negative bacteria that add or remove acyl chains or phosphate groups or add chemical groups such as

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The abbreviations used are: TLR, Toll-like receptor; LOS, lipooligosaccharides; ANOVA, analysis of variance; HA, hemagglutinin; MPLA, monophosphoryl lipid A.
Bordetella pertussis is a Gram-negative bacterium that causes whooping cough in humans. We previously showed that B. pertussis strain BP338 can modify its lipid A by the addition of glucosamine moieties. Glucosamine modification increases activation of TLR4-MD-2 in human, but not mouse, cells (15, 17, 18). Specifically, we previously showed using human THP-1 cell-derived macrophages that the production of several proinflammatory cytokines (e.g. IL-6, TNFα) was attenuated in macrophages stimulated with non-glucosamine-modified mutant B. pertussis (15). However, this was not the case with mouse RAW 264.7 macrophages, which mounted robust cytokine responses to the glucosamine mutant (15). Thus, cytokine production in response to glucosamine modification of B. pertussis lipid A was affected in a host-specific manner. Furthermore, because transiently transfected HEK293 cells expressing human, but not mouse, TLR4-MD-2 responded poorly to unmodified B. pertussis lipid A in NFκB reporter assays (15), we narrowed the difference between human and mouse cell responses down to the TLR4-MD-2 receptor complex.

We and others also previously observed that penta-acylated B. pertussis lipooligosacharides (LOS) are poorly stimulatory to human macrophages and dendritic cells compared with hexa-acylated E. coli LPS (19, 20), including rough LPS, which lacks the O-antigen (20). This is similar to what others have reported for tetra-acylated lipid IVA, which was described as a precursor for the O-antigen (20). This is similar to what others have reported. This is furthering the understanding of how lipid A variants from different bacterial species can have agonist or antagonist activity, depending on whether the responding host cells express human or murine TLR4-MD-2 (12–16).

We show that penta-acylated B. pertussis lipid A also responds poorly to unmodified B. pertussis lipid A in NFκB reporter assays (15), which was described as a precursor structure during the biosynthesis of hexa-acylated E. coli lipid A (3). Lipid IVA is an antagonist for human TLR4-MD-2, although it is a good agonist for mouse TLR4-MD-2 (21). The significance of host-specific effects on TLR4-MD-2 activation has been described for tetra-acylated lipid A from Yersinia pestis; mice expressing human TLR4-MD-2 displayed low levels of proinflammatory cytokines, were impaired in their ability to clear the bacteria, and exhibited decreased survival after infection compared with wild type mice, which expressed murine TLR4-MD-2 (14, 22).

Using the available crystal structure data (4, 5, 23) for TLR4-MD-2 in complex with E. coli lipid A structures (both hexa-acylated and lipid IVA), we dissected the mechanism by which human and mouse TLR4-MD-2 receptor complexes differentially respond to B. pertussis lipid A both with and without the glucosamine modification. Some amino acids in TLR4 and MD-2 that engage tetra-acylated E. coli lipid IVA were also important for the recognition of penta-acylated B. pertussis lipid A. However, we also identified novel amino acids that are specific for responses to B. pertussis lipid A. We speculate that furthering the understanding of how lipid A variants from important human pathogens are recognized in a host-specific manner by TLR4-MD-2 will provide insight into how these pathogens manipulate host immune responses and may additionally aid in the development of improved vaccine adjuvants targeted to engage specific parts of the receptor.

**Experimental Procedures**

**Cell Line**—Human embryonic kidney 293 (HEK293) cells were from ATCC and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum (Sigma) and GlutaMAX (Life Technologies) in the presence of 50 units/ml penicillin and 50 μg/ml streptomycin (Life Technologies). Cells were passaged by incubating in 0.25% trypsin/EDTA (Life Technologies) for 5 min at room temperature.

**Reagents**—E. coli (K12) LPS (InvivoGen), lipid IVA (Peptides International), and Pam3CSK4 (EMC Microcollections) were obtained, aliquoted, and stored at −20 °C. Purified LOS from B. pertussis (BP338 or the “glucosamine mutant” (18)) was obtained from Martine Caroff, as described in Shah et al. (18). DNA was isolated using the Endofree plasmid maxi kit (Qiagen). Plasticware used was guaranteed endotoxin-free.

**Heat-killed Bacteria**—B. pertussis BP338, a derivative of Tohama I, was obtained from Alison Weiss (University of Cincinnati). The isogenic BP338 glucosamine mutant was previously described in Refs. 15 and 18. Bacteria were grown on Bordet-Gengou agar (BD Biosciences) supplemented with 15% sheep blood (Dalynn) for 72 h, then in Stainer-Scholte broth containing 10.72 g/liter glutamate and 0.24 g/liter proline and supplemented with 0.15% bovine serum albumin (Sigma). Bacteria were then resuspended in phosphate-buffered saline to an absorbance at 600 nm (A600) of 5. This is equivalent to roughly 7.5 × 10⁸ cfu/ml. Bacteria were then heat-killed by incubation at 56 °C for 1 h and used at a final concentration of 7.5 × 10⁹ cfu/ml in reporter assays.

**Generation of Mutants**—Mutants of TLR4 and MD-2 were generated by overlap extension PCR (24). PCR fragments were then subcloned into HA-tagged wild type TLR4 in pDisplay (13) and FLAG-tagged wild type MD-2 in pEFBOS (K. Miyake) vectors. Primer sequences for the generation of these mutants are available upon request.

**Transfection, ELISA, and Reporter Assays**—HEK293 cells were seeded at 4 × 10⁴ cells per well in 96-well flat-bottom Falcon tissue culture plates (Fisher) and incubated overnight at 37 °C in 5% CO₂. The next day cells were transfected with 0.1 μg of total plasmid DNA consisting of pDisplay hemagglutinin (HA)-tagged TLR4 in pDisplay (13) and FLAG-tagged wild type MD-2 in pEFBOS (K. Miyake) vectors. Primer sequences for the generation of these mutants are available upon request.

**Transfection**—Cells (1 × 10⁵) were transfected with 0.1 μg of total plasmid DNA consisting of pDisplay hemagglutinin (HA)-tagged TLR4 in pDisplay (13) and FLAG-tagged wild type MD-2 in pEFBOS (K. Miyake) vectors. Primer sequences for the generation of these mutants are available upon request.

**ELISA**—Reagents—E. coli (K12) LPS (InvivoGen), lipid IVA (Peptides International), and Pam3CSK4 (EMC Microcollections) were obtained, aliquoted, and stored at −20 °C. Purified LOS from B. pertussis (BP338 or the “glucosamine mutant” (18)) was obtained from Martine Caroff, as described in Shah et al. (18). DNA was isolated using the Endofree plasmid maxi kit (Qiagen). Plasticware used was guaranteed endotoxin-free.

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strains of *B. pertussis* (BP338 and glucosamine mutant) or media alone (unstimulated) in a final volume of 100 µl for 4 h. For analysis of cytokine production, cells were transfected with TLR4/MD-2/CD14 constructs as for reporter assays plus empty vector DNA (up to 0.1 µg total DNA). Cells were stimulated the next day with purified LOS from BP338 or the glucosamine mutant or from unstimulated controls containing diluted DMSO only (as LOS was reconstituted in DMSO) at 10 ng/ml. After 24 h, supernatants were collected, and IL-8 production was measured by ELISA according to the manufacturer’s directions (Biologend). For reporter assays, the cells were lysed with 50 µl of 1× passive lysis buffer (Promega), and 10 µl of this was analyzed for reporter activity using the dual luciferase reporter assay system (Promega) on a Varioskan Flash or Fluoroskan plate reader (Thermo Scientific). NFKB luciferase reporter activity was normalized to constitutive β-actin reporter activity within each sample. Each condition was performed in triplicate in every experiment, and data from at least three independent experiments were analyzed. Statistical analysis was performed in Graph Pad Prism 6 using two-way ANOVA with repeated measures and Sidak or Tukey post-tests. For reporter assays, before ANOVA, data were transformed to log scale to correct for heteroskedasticity. p values of <0.05 were considered statistically significant.

**Detection of Proteins by Western Blot**—HEK293 cells, seeded at 4 × 10^4/well in duplicate wells, were transfected with 0.1 µg of total DNA consisting of pDisplay HA-tagged TLR4 constructs plus empty pDisplay vector using Lipofectamine LTX. Cells were lysed the following day in 25 µl of 1× passive lysis buffer, and pooled duplicate wells were heated to 37 °C for 15 min then run on a 6% polyacrylamide gel under reducing conditions and transferred to a PVDF membrane. Membranes were dried overnight then incubated in HA.11 antibody, clone 16B12 (Covance), diluted 1/1000 in 5% skim milk in Tris-buffered saline (pH 7.5) + 0.1% Tween 20 (herein called TBST buffer) for 1 h with shaking at room temperature. Membranes were washed in TBST buffer briefly, then incubated in goat anti-mouse secondary antibody conjugated to horseradish peroxidase (Jackson Immunoresearch Laboratories) diluted 1/10,000 in 5% skim milk/TBST for 1 h at room temperature with shaking. Membranes were washed 5 times for 5 min each, and then proteins were visualized using Luminata crescendo HRP substrate (Millipore). For detection of β-actin, the membrane was blocked with 5% skim milk in TBST for 1 h, then re-probed with anti-β-actin antibody, clone BA3R (UBC Antibody Laboratory) diluted 1/2,000 in 5% skim milk in TBST, followed by goat anti-mouse secondary antibody conjugated to horseradish peroxidase (Jackson Immunoresearch Laboratories) diluted 1/10,000 in 5% skim milk in TBST. Membranes were washed as before, and then proteins were visualized using Western Lightning chemiluminescence reagent (PerkinElmer).

**Results**

**The Middle 330 Amino Acids of TLR4, Along with MD-2, Coordinate Host-specific Responses to B. pertussis**—It is not known how *B. pertussis* lipid A engages TLR4-MD-2. To address the mechanism by which this lipid A is recognized, we took a systematic approach by taking advantage of the TLR4-MD-2 crystal structures available for *E. coli* LPS and lipid IVA. We hypothesized that the differences in NFKB activation by TLR4-MD-2 in response to a mutant strain of BP338, which lacks glucosamine modification, were due to differences in the amino acid sequences of human and mouse TLR4 or MD-2. To test this, we expressed wild type and mutant TLR4-MD-2 molecules, which exchange specific regions or amino acids between the human and mouse receptors, in HEK293 cells and screened for TLR4 activation using NFKB reporter assays previously used for dissecting *E. coli* lipid A structures.

We used heat-killed bacteria to stimulate the cells; therefore, we performed control experiments to titrate the concentration of bacteria used to stimulate the cells in this assay and tested untransfected HEK293 cells to ensure that the response is TLR4-dependent. Stimulation of the cells transfected with wild type human or mouse TLR4-MD-2/CD14 with ∼7.5 × 10^6 cfu/ml (1/10,000) was sufficient to induce optimal NFKB responses (Fig. 1 and data not shown). We confirmed that heat-killed bacteria from BP338 did not stimulate NFKB responses in cells that were not transfected with TLR4 (No TLR in Fig. 1) and only transfected with MD-2, CD14, and NFKB vector and β-actin Renilla luciferase reporter constructs. To confirm that the reporter constructs were working, we also tested positive controls where cells were transfected with TLR4-MD-2/CD14 or TLR1-TLR2-CD14 and stimulated with heat-killed pertussis strains. In this case we could detect robust signaling to NFKB (Fig. 1).

To narrow down a region that is important for host-specific responses to *B. pertussis*, we first tested the importance of a 330-amino-acid region of the extracellular domain of TLR4, which mediates species-specific responses to *Pseudomonas aeruginosa* LPS (13). Thus, we compared receptor chimeras (shown in Fig. 2, A and B) that interchange the middle 330 amino acids in the extracellular domain of TLR4 between the human and mouse receptors for their ability to activate an NFKB reporter in response to the different *B. pertussis* strains.
First, we confirmed similar expression levels of the various TLR4 chimeras and mutants used in this study by Western blot (Fig. 2C). Transfections of all mutants used in this study were optimized to yield comparable levels of protein expression as wild type controls by Western blot. Additionally, we confirmed host-specific responses to lipid IVA (cells expressing human TLR4-MD-2 respond poorly, but cells expressing mouse TLR4-MD-2 activate NFκB robustly) as shown by others (13, 25–27) and equal activation in response to *E. coli* LPS (Fig. 2D).

As shown in Fig. 2E and as we have seen previously (15), cells expressing human TLR4-MD-2 activated NFκB significantly better in response to wild type BP338 compared with the glucosamine mutant, whereas in cells expressing mouse TLR4-MD-2, there was no difference in NFκB in response to the two strains. Exchanging the middle 330 amino acids in the extracellular domain of TLR4 (HHM and MHM) and co-expressing MD-2, which is the same species as the middle part of TLR4 (HHM + mouse MD-2, MHM + human MD-2) exchanged

![FIGURE 2. Importance of middle 330 amino acid residues in TLR4 for host-specific responses to *B. pertussis*.](image-url)
host-specific responses to lipid IVA (Fig. 2D), as shown previously (13) and also in response to the glucosamine mutant (Fig. 2E). Similar to wild type human TLR4, cells expressing the mouse TLR4 receptor in which the middle part has been substituted with human TLR4 (MHM), exhibited an attenuated NFκB response to the glucosamine mutant compared with wild type BP338. Conversely, for cells expressing human TLR4 with the middle part of mouse TLR4 (HMH), there was no significant difference in the NFκB response to wild type or the glucosamine mutant, which was the same as for cells expressing wild type mouse TLR4.

However, when the HMH TLR4 chimera was co-expressed with human MD-2, there was a significant difference in the response to BP338 as compared with the glucosamine mutant (Fig. 2E), and the overall response to both B. pertussis strains was suboptimal compared with when HMH was co-expressed with mouse MD-2. The NFκB response was further attenuated in the HMH TLR4 chimera co-expressed with mouse MD-2 (Fig. 2F). This observation was consistent with previous data that co-expressing a human TLR4 with mouse MD-2 or mouse TLR4 with human MD-2 leads to poor activation of NFκB in reporter assays (15). This suggests that TLR4 and MD-2 both coordinate the response to B. pertussis lipid A.

Although the middle 330 amino acid region of the extracellular portion of TLR4 was sufficient to mediate species-specific responses, we could not rule out the possibility that the intracellular portion of TLR4 may also play a role. Therefore, we screened mutants of TLR4 in which the intracellular domains were interchanged between human and mouse receptors (13). Similar to wild type receptors, cells expressing a human TLR4 receptor with a mouse intracellular domain exhibited attenuated NFκB responses to the glucosamine mutant. Cells expressing a mouse TLR4 receptor with a human intracellular domain were found to be slightly less responsive to BP338 with the glucosamine modification compared with the wild type (mouse) receptor, although not to the same extent as a wild type human receptor, which showed a 2-fold reduction in NFκB activity upon stimulation with the glucosamine mutant (Fig. 2F). Thus, the human intracellular domain of TLR4 may also make a partial contribution toward species-specific responses.

Substitution of Glu-369 and Gln-436 in Human TLR4 with Positively Charged Amino Acids Rescues the Response to the Glucosamine Mutant—To narrow down which amino acids in this region are important for species-specific responses to B. pertussis lipid A, we first considered how non-glucosamine-modified lipid A might engage the human and mouse receptors (Fig. 2, G and H). Work from other groups (4, 25, 28) has shown that positively charged amino acids in the extracellular domain of TLR4 are critical for species-specific responses to lipid IVA. Specifically, Ohto et al. (4) identified several positively charged amino acids in mouse TLR4 that are not present in human TLR4 and which engage the 1- and 4′-phosphate groups of lipid IVA. Although it is not known how penta-acylated structures are oriented within the MD-2 pocket, we initially selected mutants for screening based on the interaction of human and mouse TLR4-MD-2 with lipid IVA, as non-glucosamine-modified B. pertussis lipid A, like lipid IVA, is a good agonist for the mouse, but not the human, receptor.

Positively charged amino acids that are unique to mouse TLR4 and that engage the 1-phosphate group on lipid IVA include Lys-341 on TLR4 and Lys-367* and Arg-434* on TLR4* (the second TLR4 molecule in the dimerized TLR4-MD-2 receptor). The 4′-phosphate group is surrounded by Arg-266, Lys-319, and Arg-337 (4). First, we focused on Lys-367 and Arg-434 in mouse TLR4*. These amino acids engage the phosphate group on lipid IVA when it is anchored in mouse MD-2 (depicted in the orange oval in Fig. 2H). Mutation of human TLR4 to incorporate these positively charged amino acids (Glu-369 in human mutated to Lys, Gln-436 mutated to Arg) and co-expression of mouse MD-2 were shown to rescue the response to lipid IVA (25). The introduction of these positively charged amino acids is expected to affect the recognition of lipid A molecules by engaging the negatively charged phosphate group on lipid A at the dimerization interface (Fig. 2, G and H, orange oval). We hypothesized that the introduction of Lys and Arg at positions 369 and 436 in human TLR4 might also rescue the response to non-glucosamine-modified BP338.

We thus expressed the E369K/Q436R mutant of human TLR4 in HEK293 cells along with either human or mouse MD-2 and screened for NFκB activity after stimulation with E. coli LPS or lipid IVA, BP338, or the glucosamine mutant (Fig. 3A). As shown in Fig. 3A, the E369K/Q436R mutant human TLR4 displayed equivalent NFκB activation in response to the glucosamine mutant as wild type BP338. Interestingly, and in contrast to lipid IVA, this rescue of the response to the glucosamine mutant was not dependent on co-expression of mouse MD-2. This suggests that BP338 lipid A is positioned in both human and mouse MD-2 in such a way that the phosphate group on lipid A can engage Lys-369 and Arg-434 on the human TLR4 receptor. For lipid IVA, co-expression of mouse MD-2 is needed to position lipid IVA slightly up and out of the pocket so that the phosphate groups can “reach” the positively charged amino acids introduced into human TLR4* (4, 25).

On the other hand, the reverse mutations in the mouse TLR4 receptor (K367E and R434Q) did not significantly affect the response to the glucosamine mutant (Fig. 3B), which was again true regardless of the species of MD-2 co-expressed. As substitution of the entire middle region of mouse TLR4 with human TLR4 does recapitulate a human-like response (i.e. sensitivity to the absence of glucosamine), there may be other amino acids within this region that can compensate for the loss of Lys-367 and Arg-434. In contrast to work by others (25), we did not notice an effect of the K367E/R434Q double mutant on the ability of the mouse TLR4-MD-2 receptor to respond to lipid IVA at both 10 ng/ml (Fig. 3B) or 100 ng/ml (data not shown) when the double mutant was co-expressed with mouse MD-2. The response to BP338 and the glucosamine mutant by the K367E/R434Q double mutant of mouse TLR4 was attenuated in the presence of human MD-2 (Fig. 3B).

We additionally wanted to determine whether these mutations would affect downstream outputs, including cytokine production. We stimulated HEK293 cells transfected with E369K/Q436R human TLR4 and mouse MD-2 with purified LOS from wild type BP338 or GlcN mutant strains. Similar to NFκB activity, IL-8 production was attenuated in response to GlcN-LOS in cells that expressed human, but not mouse,
TLR4-MD-2. However, mutation of Glu-369 and Gln-436 in human TLR4 to Lys and Arg rescued IL-8 production in response to GlcN-LOS. Transfected cells were also stimulated with *E. coli* LPS at 10 ng/ml and produced similar amounts of IL-8 regardless of the species of TLR4 expressed (data not shown). Because the IL-8 results mirrored what we were seeing with the NFκB screening, we hereafter screened other mutants using only NFκB reporter activity.

**Interchanging Other Unique, Charged Amino Acids between Human and Mouse TLR4 Did Not Exchange Species-specific Responses to the Glucosamine Mutant**—As mutation of Lys-367 and Arg-434 to Glu and Gln (found in human) did not affect the ability of the mouse receptor to activate NFκB in response to stimulation with the glucosamine mutant, we also tried mutating Lys-341, which is found in TLR4 and should also engage the 1-phosphate group on lipid A. Replacement of Lys-341 with a glycine (found in human TLR4) did not change the insensitivity of the mouse receptor to the glucosamine modification, although overall, NFκB activation was decreased compared with wild type mouse TLR4. Conversely, the mutation of Gly-343 in human TLR4 to Lys did not significantly affect the sensitivity of the human receptor to the loss of glucosamine (Fig. 4A).

We next looked at whether introduction of positively charged amino acids in mouse TLR4, which were proposed by others (4) to engage the 4'-phosphate group on lipid IVA, could boost human responses to the glucosamine mutant (*pink residues in the blue oval in Fig. 2G, blue residues in magenta oval in Fig. 2H*). We found that the introduction of Arg at position 267 did not increase the NFκB response to the glucosamine mutant (Fig. 4B). Furthermore, mutating Glu-321 and Asn-339 in human TLR4 to Lys and Arg, found in the mouse receptor, alone or in combination with G267R did not completely rescue the defect in NFκB activation in response to the glucosamine mutant, although the differences between BP338 and the mutant were no longer significant (Fig. 4C).

We additionally mutated Ser-386 in mouse TLR4* to Lys (which is found at the corresponding position in human TLR4*), as Lys-388 was suggested to engage the 1-phosphate on hexa-acylated lipid A by Park et al. (5). However, the S386K mutation did not affect NFκB signaling downstream of *E. coli* LPS, lipid IVA, BP338, or the glucosamine mutant (Fig. 4D). We also made the reverse K388S mutation in human TLR4 and did not find this mutation to significantly affect the NFκB response to *E. coli* LPS, lipid IVA, or the pertussis strains (Fig. 4E). Therefore, we concluded that the species-specific responses to non-
glucosamine-modified pertussis lipid A are primarily due to the absence of positively charged Lys-367 and Arg-434 in human TLR4* at the dimerization interface.

Multiple Negatively Charged Amino Acids in Human TLR4 Are Important for Recognition of BP338—We next asked which amino acids in human TLR4 might engage glucosamine-modified BP338 and allow more robust responses to wild type BP338 compared with the glucosamine mutant. We hypothesized that there might be negatively charged amino acids on TLR4* that engage positively charged glucosamine-modified BP338 but not the negatively charged phosphate groups on unmodified BP338. Using the crystal structure models available for TLR4-MD-2 in complex with E. coli lipid A and lipid IVA, we identified several candidate negatively charged residues in the vicinity of Glu-369 and Gln-436, which might engage glucosamine-modified phosphate groups on B. pertussis lipid A (Fig. 2G, negatively charged residues are red and contained within the orange oval).

Single point mutations of Glu-369, Asp-371, Asp-395, or Glu-439 changed to alanine to neutralize the negative charge did not significantly affect the NFκB response to wild type BP338 or glucosamine mutant (GlcN- mutant) strains or E. coli LPS or lipid IVA or unstimulated, as controls. Two-way ANOVA was performed with a Tukey post-test, and only significant differences between BP338 and glucosamine mutant are depicted in the figure. ***, p < 0.001; **, p < 0.01; *, p < 0.05. Data are an average of 3–5 experiments; error bars indicate S.E.
of the receptor to signal. To further investigate this second possibility, we generated various combinations of mutations of two, three, or four amino acid residues and screened for NFκB responses. Both the E369A/D371A and D395A/E439A double mutants had a significantly attenuated response to the glucosamine mutant compared with wild type BP338, similar to wild type human TLR4 (Fig. 5B). Similarly, triple mutation E369A/D371A/E439A (EDE, Fig. 5C) of human TLR4 showed significantly attenuated NFκB activity in response to the glucosamine mutant. However, NFκB activity in cells expressing the quadruple mutant E369A/D371A/D395A/E439A was not significantly different in response to wild type BP338 and the glucosamine mutant (Fig. 5C, labeled EDDE), suggesting that multiple negatively charged amino acid residues are important for the ability of human TLR4 to discriminate between glucosamine modified and unmodified B. pertussis lipid A. There was no effect of any of these mutations on the response to E. coli LPS and lipid IVA (Fig. 5, A, B, and C), pointing to the specific importance of these residues for responses to B. pertussis lipid A. Furthermore, the collective contribution of multiple negatively charged residues suggests that minor but specific polymorphisms or amino acid changes to the TLR4 receptor can allow for a gradient of levels of receptor activation, the significance of which is increasingly appreciated (9).

We also mutated other negatively charged or polar amino acids at the dimerization interface to determine whether the effect we were seeing was specific: these included Glu-422, Ser-392, and Ser-394 (Fig. 6A). When these were mutated to alanine in combination with the triple mutant E369A/D371A/E439A, we did not observe a loss of the ability to discriminate between wild type BP338 and the glucosamine mutant (GlcN mutant). However, the E422A/E369A/D371A/D395A/E439A mutant (Mut 2) resulted in attenuated NFκB responses to both BP338 and the glucosamine mutant, suggesting that there is some contribution of Glu-422 in the response to BP338, irrespective of the glucosamine modification.

Amino Acids in MD-2 Contribute to Host-specific Responses to B. pertussis—We also generated point mutations in MD-2 to determine the contribution of these to signaling in response to B. pertussis strains. Others have shown that Lys-122 and Lys-125 in human MD-2 (Glu-122 and Leu-125 in mouse MD-2)
TLR4 Mutants That Affect Recognition of B. pertussis Lipid A

A crystal structure of the human TLR4-MD-2 receptor at the dimerization interface, adapted from Park et al. (5), showing the location of additional negatively charged or polar amino acids Glu-422, Ser-392, and Ser-394. These were mutated to alanine in combination with previously mutated amino acids from Fig. 5 and screened for NFκB activation. B, human (h) TLR4 with multiple point mutations were expressed in HEK293 cells, and cells were stimulated with E. coli LPS, lipid IVA, BP338, or glucosamine mutant (GlcN mutant). Mut 1 = E369A/D371A/E422A/E439A; Mut 2 = E369A/D371A/D395A/E422A/E439A; Mut 3 = E369A/D371A/E423A/S392A/S394A. The graph shows an average of three experiments; error bars indicate S.E. Two-way ANOVA was performed with a Tukey post-test, and only significant differences between BP338 and the glucosamine mutant are depicted in the figure. *, p < 0.05.

In the case of pertussis lipid A, we had observed that the introduction of positively charged residues in human TLR4* at position 369 and 436 increased signaling in response to the non-glucosamine modified mutant irrespective of the species of MD-2 we co-expressed (Fig. 3A). Therefore, we did not expect to see an effect of the K122E or K125L mutation in human MD-2. Although we did not note a significant effect of the mutation of K122E in human MD-2 on the ability to discriminate between wild type BP338 and glucosamine mutant (the difference is still statistically significant), overall, the response to both strains of pertussis was attenuated (Fig. 7A, top panel). On the other hand, substitution of Lys-125 in human MD-2 with Leu rescued the response to the glucosamine mutant (Fig. 7A, bottom panel) in addition to lipid IVA. This occurred when the mutant MD-2 was co-expressed with wild type human TLR4, suggesting a different mechanism than that seen for lipid IVA. The reverse mutations (E122K and L125K) in mouse MD-2 did not affect the response to BP338 or the glucosamine mutant (Fig. 7B).

Others have suggested that Leu-125 and Pro-127 in mouse MD-2 contact Leu-442 in mouse TLR4*. As this leucine is conserved in the human TLR4 (Leu-444) receptor, it is possible that the introduction of Leu-125 into human MD-2 creates an additional interface to promote receptor complex dimerization (Fig. 2G, green oval). Although the introduction of Leu-125 was found to promote NFκB activation in response to the glucosamine mutant, the introduction of a proline residue to replace Ser-127 in human MD-2 did not have the same effect (Fig. 7C). The S127P mutant of human MD-2 decreased the NFκB response to both wild type and the glucosamine mutant, suggesting that the expression of serine at this position is important for the ability of the human receptor to respond to pertussis lipid A. The reverse mutation in mouse MD-2 (P127S) did not have an effect on either wild type or GlcN-BP338 (Fig. 7D).

Uncharged Phenylalanine Residues in TLR4 Are Critical for Responses to B. pertussis—Previous work by others has shown that uncharged amino acids in TLR4* are also important for the recognition of hexa-acyl E. coli LPS by human TLR4, possibly by engaging the acyl chain that sticks out of the MD-2 pocket, or uncharged amino acids on MD-2 (5, 7). To determine whether this interface is also important for responses to B. pertussis lipid A, we generated mutants in human and mouse TLR4 at Phe-440, Leu-444, and Phe-463 (human) and Phe-438, Leu-442, and Phe-461 (mouse) as shown in Fig. 2, G and H, and assayed for NFκB responses to B. pertussis strains. Mutation of phenylalanine residues in human (F440A and F463A; Fig. 8A, top and bottom panels) and mouse (F438A and F461A; Fig. 8B, top and bottom panel) TLR4 abolished the ability of both receptors to respond to either BP338 or the glucosamine mutant. Therefore, these phenylalanine residues are critically important for responses to B. pertussis.

Similar to Resman et al. (7, 8), we observed species-specific differences in the ability of human and mouse TLR4 mutants to respond to E. coli LPS and lipid IVA. F440A or F463A mutation of human TLR4 caused a more severe phenotype: the complete or near-complete loss in the ability to activate NFκB in response to both E. coli LPS and lipid IVA, whereas F438A or F461A mutants of mouse TLR4 could still partially respond to E. coli LPS (Fig. 8, A and B). Mutation of leucine at position 442 (mouse) or 444 (human) had a slightly more conservative effect, with the mouse TLR4 receptor able to respond weakly to B. pertussis strains and lipid IVA and robustly to E. coli LPS and the human TLR4 receptor, although unable to respond to B. per-
tussis strains, still able to respond to E. coli LPS (Fig. 8, A and B, middle panels).

**Positively Charged Amino Acids in Mouse TLR4 Support Recognition of E. coli LPS**—The relative importance of these phenylalanine residues in TLR4 was host-specific for E. coli LPS. We hypothesized that this is because of the presence of the positively charged amino acids in mouse TLR4, including those at positions 367 and 434, which could perhaps support TLR4 dimerization even in the absence of one of the phenylalanine residues at the uncharged dimerization interface. We have already shown that substitution of Glu-369 and Gln-436 in the human receptor with Lys and Arg rescued the ability of the human receptor to respond to non-glucosamine modified BP338 lipid A (Fig. 3A). Thus, we generated triple mutants E369K/Q436R/F440A and E369K/Q436R/F463A in human TLR4 and screened for NFκB activity in response to E. coli LPS. As shown in Fig. 9A, the introduction of positively charged amino acids at positions 369 and 436 partially rescued the ability of human TLR4 with the F463A mutation to respond to E. coli LPS. The F440A mutation was not fully rescued by the introduction of these positive residues, but the NFκB signal in response to E. coli LPS for the triple mutant was still significantly greater than the unstimulated control, unlike the F440A mutation alone. Conversely, in the mouse TLR4 receptor, mutation of Lys-367 and Arg-434 to Glu and Gln, respectively, combined with the mutations F438A or F461A essentially abolished NFκB activity in response to E. coli LPS, suggesting that these positively charged residues contribute to the activation of...
mouse TLR4 in response to *E. coli* LPS (Fig. 9B). However, the introduction of positively charged amino acids to human TLR4 does not rescue the response to *B. pertussis* strains (Fig. 9A), consistent with the data that show that the mouse receptor with F438A or F461A mutation also does not activate NFκB response to *B. pertussis* (Fig. 8B).

Taken together, we interpret these findings to mean that both charged and uncharged interactions can contribute to the ability of TLR4-MD-2 to respond to *E. coli* LPS. In the case of human TLR4, loss of Phe-440 or Leu-444 alone was sufficient to abrogate the response to *E. coli* LPS, but mouse TLR4, possibly due to the contribution of charged interactions at position 367 and 434, can tolerate single mutations to either Phe-438 or Phe-461. Importantly, uncharged interactions are crucial for both human and mouse receptors to respond to *B. pertussis* LPS irrespective of the presence or absence of the glucosamine modification, as mutation of any, including the more conservative leucine to alanine mutation, results in attenuated or absent NFκB responses to BP338.

We hypothesized that the reason we still observed a robust NFκB response to *E. coli* LPS in the human F440A/mouse F438A or human F463A/mouse 4461A mutants, but none to *B. pertussis* strains, was because of the sixth acyl chain of *E. coli* lipid A, which is absent in BP338. At this uncharged interface, Phe-440* and Phe-463* in TLR4* in a dimerized receptor complex can interact with uncharged amino acid residues in MD-2 (Val-82, Met-85, and Leu-87, shown in the green oval in Fig. 2G) and a portion of one of the secondary acyl chains of hexa-acylated lipid A, which does not fit in the MD-2 pocket (4, 5, 7). It is not clear whether the acyl chains of penta-acylated pertussis lipid A are exposed on the surface of MD-2 or completely buried inside the pocket. We reasoned that if the acyl chains are buried inside the pocket, then loss of Val-82/Met-85/Leu-87 in MD-2, similar to loss of Phe-440/Phe-463 in TLR4, should result in the attenuation of NFκB signaling in response to *B. pertussis* strains.

To test this, we generated the V82I/M85I/L87F mutant of human MD-2 described previously by Resman et al. (7). As
shown in Fig. 10, whereas this mutant still generates a fairly robust NFκB response to E. coli LPS, the response to BP338 and the glucosamine mutant is severely compromised.

Our results, although not conclusive, lend support to the model that penta-acylated B. pertussis engages TLR4-MD-2 in a manner more similar to tetra-acylated lipid IVA than hexa-acylated E. coli lipid A.

Taken together, our data present some conserved, and some novel, amino acid residues that are important for recognition of penta-acylated B. pertussis lipid A and furthermore identify the basis for host-specific responses to glucosamine modification of lipid A found in B. pertussis strain BP338.

**Discussion**

Here, we identified key amino acid residues by which TLR4-MD-2 recognizes glucosamine modified and unmodified B. pertussis lipid A in a host-specific (human versus mouse) manner. Previous studies have demonstrated a model for how lipid A structures from E. coli LPS (6-acyl lipid A and its precursor lipid IVA) are recognized by human and mouse TLR4-MD-2 (4, 5, 23, 25–29). However, work over the past two decades has revealed a wide spectrum of possible lipid A structures from various relevant and important human pathogens (11, 30).

Surprisingly, although some features of lipid A recognition are conserved between B. pertussis and E. coli, there are also several significant differences. First, the introduction of positively charged amino acids Lys-369 and Arg-436 into human TLR4 was sufficient to rescue the response to non-glucosamine modified lipid A from BP338; this rescue was not dependent on co-expression of mouse MD-2, which was important for recognition of lipid IVA. Thus, this implies that the lipid A from B. pertussis is positioned in MD-2 in such a way that the negatively charged phosphate groups on unmodified lipid A are able to contact the positively charged amino acids in TLR4. In support of this, the increase in TLR4 activation by mutating Lys-125 to Leu in human MD-2 occurs independently of TLR4.

Secondly, the presence of negatively charged amino acids in human TLR4 negatively regulates signaling in response to unmodified B. pertussis lipid A; however, the effect of mutating these to neutral alanines was specific to the glucosamine-deficient strain of BP338; this rescue was not dependent on co-expression of mouse MD-2, which was important for recognition of lipid IVA. Thus, this implies that the lipid A from B. pertussis is positioned in MD-2 in such a way that the negatively charged phosphate groups on unmodified lipid A are able to contact the positively charged amino acids in TLR4. In support of this, the increase in TLR4 activation by mutating Lys-125 to Leu in human MD-2 occurs independently of TLR4.

The third finding from this work is the critical importance of uncharged amino acids in TLR4 (Phe-440/Leu-444/Phe-463 in the human receptor and Phe-438/Leu-442/Phe-461 in the...
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mouse receptor) for signaling to NFκB in response to stimulation with B. pertussis. Notably, these amino acids are not only conserved between human and mouse but are also highly conserved across 23 species within the subfamily Murinae (31). Furthermore, although introduction of positively charged amino acids to replace Glu-369 and Gln-436 in human TLR4 rescued signaling through the F463A mutant receptor in response to E. coli LPS, it was not sufficient to rescue the response to B. pertussis, consistent with the fact that the mouse TLR4 receptor, when Phe-461 is mutated to Ala, does not allow for activation of NFκB in response to B. pertussis, although it does for E. coli LPS.

With regard to receptor dimerization, the host-specific discrimination of the glucosamine modification is affected at the charged interface (orange oval in Fig. 2, G and H). Receptor interactions at the other interface (green oval) are optimal with 6 acyl chains, and this is possibly potentiated by the sixth acyl chain; this could affect the potency of the overall dimerization independent of the glucosamine affects. In support of this, we additionally found that mutating Val-82/Met-85/Leu-87 in MD-2, originally described in Resman et al. (7), significantly attenuates signaling in response to B. pertussis strains but not E. coli LPS.

Recently, Needham et al. (9) demonstrated that subtle changes in the structure of lipid A from E. coli can have a wide range of effects on NFκB signaling, implying that the activation of TLR4 is not limited to simple on/off states but occurs on a gradient of activation levels. Our work has demonstrated that the different levels of activation we see with respect to the presence or absence of glucosamine, where the glucosamine mutant stimulates partial, but not completely abrogated, NFκB signaling in the human receptor, is likely dependent on a combination of interactions involving various amino acids in the TLR4-MD-2 receptor. Furthermore, the species specificity of this response suggests that host species may have co-evolved with pathogens in their environment to differentially recognize variant lipid A structures.

We have to some extent teased apart the relative contribu-
tion of the charged and uncharged amino acids toward NFκB signaling, which will further the understanding of how lipid A variants are recognized by TLR4-MD-2 and could open up avenues toward the rational design of vaccine adjuvants. However, the downstream effect of these different levels of NFκB activation and the significance of engaging one or the other part of the receptor are still unknown and merit further study. Monophosphoryl lipid A (MLPLA) from Salmonella minnesota Re595 has been shown to stimulate TRIF-dependent signaling and can effectively induce adaptive immunity (T cell proliferation and B cell antibody production) but poorly induces proinflammatory cytokines and signaling through MyD88 (32). Recent work by Chilton et al. (33) has demonstrated that monophosphorylated penta-acetylated structures of lipid A from Bacteroides thetaiotaomicron and Prevotella intermedia, when co-administered with peptide antigens, induce less CD8 T cell proliferation in an in vivo mouse model compared with hexa-acetylated monophosphoryl lipid A from S. minnesota. Further investigation using MLPLA-primed mice has revealed that poorer signaling through MyD88 is associated with the generation of fewer long term memory CD8 T cells (34). Thus, subtle manipulation of lipid A structures could have very specific effects not only on the cytokines and chemokines produced by innate immune cells but also on the type of downstream adaptive immune response elicited and points to the potential to tailor the design of LPS adjuvants for specific pathogen-induced diseases.

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