Substitution of the *Bordetella pertussis* Lipid A Phosphate Groups with Glucosamine Is Required for Robust NF- κ B Activation and Release of Proinflammatory Cytokines in Cells Expressing Human but Not Murine Toll-Like Receptor 4–MD-2–CD14[▽]

Nico Marr,^{1†} Adeline M. Hajjar,² Nita R. Shah,¹ Alexey Novikov,³ Cathy S. Yam,²
Martine Caroff,³ and Rachel C. Fernandez^{1*}

Department of Microbiology & Immunology, University of British Columbia, Vancouver, British Columbia, Canada¹; Department of Medicine, University of Washington, Seattle, Washington²; and Equipe Structure et Activités des Endotoxines, UMR 8621 du Centre National de la Recherche Scientifique, IGM, Université de Paris-Sud-XI, Orsay, France³

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Bordetella pertussis endotoxin is a key modulator of the host immune response, mainly due to the role of its lipid A moiety in Toll-like receptor 4 (TLR4)-mediated signaling. We have previously demonstrated that the lipid A phosphate groups of *B. pertussis* BP338 can be substituted with glucosamine in a BvgAS-regulated manner. Here we examined the effect of this lipid A modification on the biological activity of *B. pertussis* endotoxin. We compared purified endotoxin and heat-killed *B. pertussis* BP338 whole cells that have modified lipid A phosphate groups to an isogenic mutant lacking this modification with respect to their capacities to induce the release of inflammatory cytokines by human and murine macrophages and to participate in the TLR4-mediated activation of NF- κ B in transfected HEK-293 cells. We found inactivated *B. pertussis* cells to be stronger inducers of proinflammatory cytokines in THP-1-derived macrophages when lipid A was modified. Most notably, lack of lipid A modification abolished the ability of purified *B. pertussis* endotoxin to induce the release of inflammatory cytokines by human THP-1-derived macrophages but led to only slightly reduced inflammatory cytokine levels when stimulating murine (RAW 264.7) macrophages. Accordingly, upon stimulation of HEK-293 cells with inactivated bacteria and purified endotoxin, lack of lipid A modification led to impaired NF- κ B activation only when human, and not when murine, TLR4–MD-2–CD14 was expressed. We speculate that in *B. pertussis*, lipid A modification has evolved to benefit the bacteria during human infection by modulating immune defenses rather than to evade innate immune recognition.

Whooping cough (pertussis) is an acute respiratory illness in humans caused mainly by the strictly human pathogen *Bordetella pertussis* and less frequently by a strictly human-pathogenic branch of *B. paraperussis*. Most severe disease and almost all fatalities occur in young infants who become infected with *B. pertussis* (32).

One of the key modulators of the immune response to *B. pertussis* is its endotoxin component, a major constituent of the outer membranes of Gram-negative bacteria (4). Endotoxins of *B. pertussis* isolates are composed of a mixture of tetra- and penta-acylated lipid A moieties serving as anchors for a multi-branched dodecasaccharide core structure bearing numerous carboxyl and free amino groups and a distal trisaccharide with unusual N-acetylated sugars (5). They lack a serospecific O polysaccharide (39) and are therefore often referred to as lipooligosaccharides (LOS).

Since they are unique to Gram-negative bacteria and have a

conserved architecture, endotoxins play a crucial role during recognition of microbial infection by the host immune system. Most notably, the lipid A moiety is a ligand of a germ line-encoded receptor complex composed of Toll-like receptor 4 (TLR4), MD-2, and CD14. Like for other pattern recognition receptors (PRRs), stimulation of this receptor complex leads to activation of signaling pathways that result in the induction of antimicrobial genes and release of cytokines, thereby initiating inflammatory and immune defense responses (21). It is increasingly recognized that variability in the lipid A structure, i.e., the number and length of the fatty acid moieties attached to its generally conserved glucosamine disaccharide backbone and the presence or absence of phosphate groups and further substituents, can dramatically affect the biological activity of endotoxins (6, 10, 18, 31, 33). Downstream signaling also can be influenced by CD14 and whether endotoxin is from a strain with a smooth colony morphotype (i.e., has a serospecific O polysaccharide) or a rough colony morphotype (i.e., lacks it). In the presence of CD14, both rough- and smooth-type endotoxins bind the receptor complex, resulting in the activation of both MyD88-dependent and -independent signaling pathways, whereas in the absence of CD14, only rough-type LOS binds TLR4–MD-2 and triggers MyD88-dependent signaling (22). *Bordetella* endotoxins are generally less potent activators of the TLR4–MD-2–CD14 receptor complex than canonical agonists such as lipopolysaccharides (LPS) of *Escherichia coli* 0111:B4

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Life Sciences Centre, University of British Columbia, 2559-2350 Health Sciences Mall, Vancouver, British Columbia V6T 1Z3, Canada. Phone: (604) 822-6824. Fax: (604) 822-6041. E-mail: rachelf@interchange.ubc.ca.

† Present address: Department of Pediatrics, University of British Columbia, Child & Family Research Institute, Room A5-122, 950 West 28th Avenue, Vancouver, BC V5Z 4H4, Canada.

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or *Salmonella enterica* serovar Minnesota Re 595, at least in part due to the different number of acyl chains affecting the three-dimensional shape of the lipid A moiety (7, 16, 35). Moreover, significant differences in the biological activity of *Bordetella* endotoxins can be found, which are species specific (13, 27). We have recently shown that the *B. pertussis* Tohama I derivative BP338 is capable of substituting both lipid A phosphate groups with glucosamine (GlcN), which is dependent on a gene locus encoding a *Bordetella* ArnT (formerly PmrK) glycosyl transferase ortholog (30). This locus was found to be positively regulated by BvgAS, the master regulatory two-component system of *Bordetella* protein virulence factors (9, 30). In the present study we investigated the impact of these GlcN substituents on *B. pertussis* lipid A with respect to the induction and release of inflammatory cytokines by human THP-1-derived macrophages (M ϕ) and murine RAW 264.7 macrophages and on TLR4-mediated activation of the transcription regulator NF- κ B in transiently transfected human embryonic kidney (HEK-293) cells. These are commonly used models that allow the evaluation of the biological activity and potency of TLR ligands. Our data show a much more pronounced effect of the GlcN modification than has previously been reported for *B. pertussis* (14). Moreover, we show that human TLR4 is much more discriminating than its murine counterpart in recognizing *B. pertussis* lipid A that has or lacks the GlcN substituent.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *B. pertussis* Tohama I derivative BP338 was obtained from Alison Weiss (University of Cincinnati). *B. pertussis* BP338 and its isogenic mutant BP338GlcN⁻ were grown in the presence of 30 μ g/ml nalidixic acid. Additionally, the latter strain was grown in the presence of 30 μ g/ml gentamicin in order to maintain the genomic insertion of suicide vector pEG7-BP0398. The pEG7 plasmid (8) was a gift from Peggy Cotter (University of North Carolina, Chapel Hill). *B. pertussis* strains were grown at 37°C, first on Bordet-Gengou (BG) agar (BD Biosciences) with 15% sheep blood (Dalyne) for 72 h and then after being used to inoculate Stainer-Scholte (SS) broth (45) containing 10.72 g/liter glutamate and 0.24 g/liter proline and supplemented with 0.15% bovine serum albumin (Sigma). Inoculated to give an optical density at 600 nm (OD₆₀₀) of 0.01, the *B. pertussis* liquid cultures were grown under agitation for a further 48 h. Finally, the bacteria were harvest into phosphate-buffered saline (PBS), concentrated to an OD₆₀₀ of 5 ($\sim 7.5 \times 10^9$ CFU/ml), and killed by incubation for >40 min at 56°C. Lack of viability was confirmed by spotting 50 μ l of the concentrated bacterial suspensions onto BG blood agar plates, which were incubated for at least 5 days at 37°C and then examined for the absence of bacterial growth. The heat-killed whole-cell preparations of *B. pertussis* BP338 and its isogenic mutant were stored at -80°C. *E. coli* strains DH5 α (Invitrogen) and S17-1 (44) were used for cloning and as the donor strain for conjugation, respectively. Both strains were cultured at 37°C in Luria-Bertani (LB) broth or on LB agar.

Generation of *B. pertussis* mutant BP338GlcN⁻. An internal fragment of locus BP0398 was amplified by PCR using genomic DNA of strain BP338 and primers BP0398-KOBamHI (CGCGGATCCTTCTCGTCCACGACATT TCG) and BP0398-KOEcoRI (GGAATTCAGCTGCAAGTCCGAACGGAT TCG). The PCR product was cloned into the suicide vector pEG7 (8) using BamHI and EcoRI (New England Biolabs) according to standard molecular cloning techniques (41), generating vector pEG7-BP0398. Vector pEG7-BP0398 was introduced into BP338 by conjugation. In brief, after transformation of *E. coli* S17-1 in accordance with standard techniques (41), matings between *B. pertussis* BP338 and *E. coli* S17-1 carrying pEG7-BP0398 were conducted by swabbing cells from fresh plate cultures of the donor and recipient strains at a ratio of $\sim 10:1$ onto SS agar with no antibiotics and supplemented with 10 mM MgSO₄ and 0.15% bovine serum albumin (Sigma-Aldrich). After 5 to 7 h of incubation at 37°C, bacteria were swabbed onto BG blood agar plates containing gentamicin and nalidixic acid and incubated at 37°C for 3 to 4 days in order to select a mutant with a genomic integration of pEG7-BP0398. Genomic integration of pEG7-BP0398 into locus BP0398 was

verified by PCR using locus-specific primer BP0398rev2 (CCCCAAGCTTG GCTGTCGCTGTCTACGG) and pEG7-specific primer pEG7fw1 (TAGG CGTATCACGAGGCCCTTTC). A PCR product of 1.5 kb was indicative of the correct integration of the plasmid.

Highly purified LOS preparation. *B. pertussis* LOS preparations were extracted by an ammonium hydroxide-isobutyric acid method (6a). Primary extracts were subjected to a standard enzyme treatment (DNase, RNase, and proteinase K) and finally repurified by the acidified chloroform-methanol-water procedure as described previously (48). To be sure that no specific LOS molecular species were discriminated during the process, all intermediate and final products were analyzed by matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS). High purity of the resulting LOS preparations was evidenced by three different methods: the absence of contaminating (non-LOS) peaks was shown by positive-ion MALDI MS analysis, the absence of detectable protein contaminants was demonstrated by Tricine-SDS-PAGE (42) and silver staining (50) with loading of up to 250 ng of LOS from each preparation, and the absence of lipoprotein content was further demonstrated based on the lack of detectable levels of cysteine by analysis with an amino acid analyzer (a Hitachi L-8800 instrument equipped with a 2620MSC-PS column [ScienceTec, Les Ulis, France]).

Direct lipid A isolation from bacterial cells. For MALDI MS analysis, lipid A was isolated directly by hydrolysis of bacterial cells as previously described (11, 47). Briefly, lyophilized bacterial cells (10 mg) were suspended in 200 μ l of a mixture of isobutyric acid and 1 M ammonium hydroxide (5:3, vol/vol) and were kept for 2 h at 100°C in a screw-cap test tube with magnetic stirring. The suspension was cooled in ice water and centrifuged (2,000 $\times$ g, 10 min). The recovered supernatant was diluted with 2 volumes of water and lyophilized. The sample was then washed once with 200 μ l of methanol (by centrifugation at 2,000 $\times$ g for 10 min). Finally, lipid A was extracted from the pellet in 100 μ l of a mixture of chloroform, methanol, and water (3:1.5:0.25, vol/vol/vol).

MALDI MS analysis of LOS and lipid A samples. LOS samples were dispersed in water at 1 μ g/ μ l. Lipid A extracts in chloroform-methanol-water were used directly. In both cases a few microliters of sample solution was desalted with a few grains of ion-exchange resin Dowex 50W-X8 (H⁺), and 0.5- to 1- μ l aliquots of the solution were deposited on the target, covered with matrix solution, and allowed to dry. Dihydroxybenzoic acid (DHB) (Sigma-Aldrich) was used as the matrix. It was dissolved at 10 mg/ml in 0.1 M citric acid solution in the same solvents as those used for the analytes (46). Different analyte/matrix ratios (1:2, 1:1, and 2:1 [vol/vol]) were tested to obtain the best spectra. Analyses were performed on a PerSeptive Voyager-DE STR time-of-flight mass spectrometer (Applied Biosystems) in linear mode with delayed extraction. Negative- and positive-ion mass spectra were recorded. The ion-accelerating voltage was set at 20 kV, and the extraction delay time was adjusted to obtain the best resolution and signal-to-noise ratio.

Cell culture. HEK-293 cells were maintained in high-glucose Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum (HyClone), 2 mM GlutaMAX, 25 mM HEPES, and 50 U/ml penicillin and 50 μ g/ml streptomycin (GIBCO, Invitrogen). THP-1 and RAW 264.7 cells were maintained in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (GIBCO, Invitrogen), 2 mM GlutaMAX, and 50 U/ml penicillin and 50 μ g/ml streptomycin (GIBCO, Invitrogen). Cells were incubated at 37°C in humid air with 5% CO₂.

HEK-293 transfections and luciferase assays. HEK-293 transfections using the NF- κ B reporter construct (ELAM-1 firefly luciferase), the β -actin-*Renilla* luciferase reporter construct, the modified pDisplay expression vector, and the expression constructs for murine and human TLR4-MD-2-CD14 have been described previously (18). Amounts of transfected DNA of each construct were used as noted previously (18), except for murine TLR4 (0.0005 μ g per well) and murine MD-2 (0.005 μ g per well). All transfections were normalized to 0.05 μ g total DNA by the addition of empty vector. All TLR constructs were hemagglutinin tagged, and the amount of TLR DNA used was normalized based on relative expression from anti-hemagglutinin Western blots, as well as the equivalent *E. coli* lipopolysaccharide (LPS) response. After 3 h, the medium was replaced with fresh medium. The cells were stimulated the next day in 100 μ l medium for 4 h and then lysed with 50 μ l passive lysis buffer (Promega), and luciferase activity was measured in 10 μ l of the lysate using the dual luciferase reporter assay system (Promega).

THP-1 and RAW 264.7 stimulations and cytokine detection. For THP-1 stimulations, 5×10^5 viable THP-1 cells in 500 μ l were added to each well of tissue culture-treated, flat-bottom, nonpyrogenic, polystyrene 24-well plates (Corning Costar) and differentiated into macrophages (M ϕ) by incubation in the presence of 50 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich). After 48 h, the PMA was removed by replacing the medium twice with fresh medium, and

the cells were rested for another 72 h. The medium was then replaced with 500 μ l fresh medium supplemented with purified LOS (at 1 ng/ml, 10 ng/ml, and 100 ng/ml), heat-killed whole bacteria (1:10, 1:100, and 1:1,000 dilutions of the concentrated heat-killed whole-cell preparations at an OD₆₀₀ of 5.0, corresponding to approximately 7.5×10^9 CFU/ml), or no added stimulus. Identical plates were set up for supernatant collections, with one harvested at 4 h and one at 24 h poststimulation. Supernatants were stored in nonpyrogenic polypropylene plates (Corning Costar) at -80°C until assayed. The killed bacterial preparations were judged to not be cytotoxic based on trypan blue exclusion. For RAW 264.7 stimulations, 2×10^5 viable cells in 200 μ l were added to each well of tissue culture-treated, flat-bottom, nonpyrogenic, polystyrene 96-well plates (Corning Costar). The next day, the medium was replaced with 200 μ l of medium containing purified LOS (at 1 ng/ml, 10 ng/ml, and 100 ng/ml) or no added stimulus. Identical plates were set up for supernatant collections, with one harvested at 4 h and one at 24 h poststimulation.

Cytokines secreted by THP-1-derived macrophages upon stimulation with purified LOS were quantified with the human proinflammatory 4-Plex I and the human beta interferon (IFN- β) singleplex tissue culture kits, a Sector Imager 2400, and data analysis software from Meso Scale Discovery. For stimulations of THP-1-derived M ϕ with heat-killed whole cells, secreted cytokines were measured by means of a multiplex cytometry-based Luminex 100 system using a human cytokine 25-Plex kit (Biosource International Inc.) and StarStation data analysis software (Applied Cytometry Systems) according to the manufacturer's instructions. To quantify cytokines secreted by RAW 264.7 cells, 1:3 dilutions of the tissue culture supernatants were assayed using a Bio-Plex 200 system and Bio-Plex Pro assay kits (Bio-Rad) according to the manufacturer's instructions.

Statistical analysis. Data were analyzed using GraphPad Prism 5 software. For comparison of the data groups from the THP-1 stimulations using purified LPS, regular two-way analysis of variance (ANOVA) and linear regression analysis were performed. For all other stimulations, data groups were compared by two-way ANOVA with repeated measures. A *P* value of <0.05 was taken as a statistically significant difference.

RESULTS

Generation of an isogenic mutant of *B. pertussis* wild-type strain BP338 with free lipid A phosphate groups. We have previously demonstrated that both phosphate groups of the lipid A diglucosamine backbone of *B. pertussis* wild-type strain BP338, but not those of *B. pertussis* transposon mutant BPM2859, can be modified (30). The genetic locus disrupted in this transposon mutant encodes a *Bordetella* ArnT (formerly PmrK) glycosyl transferase ortholog which catalyzes the final step of a reaction sequence leading to the substitution of the lipid A phosphate groups of Gram-negative bacteria with carbohydrates (49). Since we found transposon mutant BPM2859 to be less hemolytic upon growth on BG blood agar (which is dependent on the bifunctional adenylate cyclase/hemolysin CyaA) and impaired in the expression of several other Bvg-regulated proteins, including BrkA (29), we generated a new *B. pertussis* mutant, BP338GlcN[−], by disrupting the gene locus BP0398 of wild-type strain BP338 with suicide vector pEG7-BP0398 via homologous recombination and confirming the insertion by PCR (data not shown). BP338GlcN[−] and its parental strain were found to be equally hemolytic upon growth on BG blood agar, and immunoblot analysis confirmed that the Bvg-regulated BrkA proteins in BP338 and BP338GlcN[−] were expressed at equal levels (data not shown), thus indicating that the BvgAS two-component system was functional in both strains. To demonstrate the presence or absence of glucosamine on the lipid A phosphate groups in the different strains, negative-ion MALDI mass spectra of lipids A and LOS isolated from wild-type BP338 and its isogenic mutant BP338GlcN[−] were compared (Fig. 1). Peaks corresponding to tetra- and penta-acyl lipids A containing glucosamine substituents (*m/z* 1494, 1720, and 1881) were found in a large amount in the lipid A spectrum from wild-type

bacteria (Fig. 1A). They were absent in the lipid A spectrum of the mutant strain, except for a minor residual peak observed at *m/z* 1720 (Fig. 1B). Spectra obtained in the positive-ion mode were used for control of fragmentation data (not shown). For both strains they revealed the presence of the prominent B1 fragment peak at *m/z* 904, corresponding to the protonated distal glucosamine (GlcN II) with its phosphate group and three fatty acids (C₁₄OH, C₁₄OC₁₄) thus confirming the proposed structures (23). The respective molecular structures established earlier (30) are given in Fig. 1E. Similarly, negative-ion MALDI mass spectra obtained from LOS of the two *B. pertussis* strains showed that the additional peaks corresponding to the LOS molecular species whose lipid A moieties are substituted with one or two GlcNs were present in BP338 (*m/z* 4014, 4217, 4378) (Fig. 1C) but absent in the mutant (Fig. 1D). A minor peak at *m/z* 1720 was again observed in the "lipid A" region of the mutant LOS mass spectrum. Comparison of the LOS mass spectra presented in Fig. 1C and D also confirms that polysaccharide moieties of LOS molecules from the wild type and its GlcN[−] mutant strain are equivalent. In both cases the same major peaks are observed in the "polysaccharide region" of the mass spectra, corresponding to the well-known dodecasaccharide structure with a proximal 3-deoxy-D-mannooctulosonic acid (Kdo) sugar substituted or not with ethanolamine-pyrophosphate or pyrophosphate derivatives (*m/z* 2496, 2453 and 2293) (30). Although small peaks at *m/z* 1790 and 1833 corresponding to the polysaccharide moiety missing the distal trisaccharide (5) were observed only in the mutant LOS spectrum (Fig. 1D), respective LOS molecular ions were visible in both strains (*m/z* 3191 and 3394). These are fairly minor molecular species that are generally expressed in a variable manner.

Lack of GlcN modification of the *B. pertussis* lipid A phosphate groups leads to impaired stimulation of proinflammatory cytokines in human THP-1-derived M ϕ in response to heat-killed bacteria. It is well established that the lipid A moiety of LPS is the ligand for the TLR—MD-2—CD14 receptor and that canonical lipid A species from *E. coli* and *Salmonella* are potent activators of inflammation. In order to elucidate whether substitution of the lipid A phosphate groups with GlcN affects the biological activity of *B. pertussis* endotoxin, we first assessed the stimulatory activity of inactivated (heat-killed) whole bacteria. Inactivated *B. pertussis* organisms serve as the antigen component for whole-cell pertussis vaccines that are still widely being used despite being more reactive than acellular pertussis vaccines (1, 2), and heat-stable *B. pertussis* TLR agonists (e.g., bacterial lipoproteins and LOS) that might be found in these preparations may influence cytokine production. Different doses of inactivated (heat-killed) whole bacteria were used, and cytokine levels were measured after stimulation for 4 and 24 h. As shown in Fig. 2, we found that heat-killed *B. pertussis* BP338 bearing the GlcN substituents induced the release of the pyrogenic cytokines interleukin-1 β (IL-1 β), IL-6, and tumor necrosis factor alpha (TNF- α) (Fig. 2A), as well as several proinflammatory chemokines such as interferon-inducible protein 10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), the monokine induced by gamma interferon (MIG), RANTES (regulated on activation, normal T cell expressed and secreted) (Fig. 2B), macrophage inflammatory protein-1 α (MIP-1 α), and MIP-1 β (Fig. 2C) and mod-

FIG. 1. (A to D) Comparison of negative-ion MALDI mass spectra of lipid A and LPS (LOS) from *B. pertussis* wild-type strain BP338 and its isogenic mutant BP338GlcN⁻. Peaks at *m/z* 1333 and 1559 represent tetra-acylated and penta-acylated species that lack the glucosamine substituent. (E) Structures of the penta-acyl molecular species without and with glucosamine substituents.

est levels of IFN- α and IFN- γ (Fig. 2D). In comparison to BP338, the isogenic mutant strain BP338GlcN⁻ generally exhibited a diminished capacity to elicit similarly high levels of these cytokines (Fig. 2). The only exception was the release of IL-1 β and perhaps IFN- α at 24 h, where differences are only minor and in the case of IL-1 β secretion after 24 h are not significant (Fig. 2). In general, the total cytokine levels induced were also found to be dose dependent. Interestingly, for many of the cytokines tested, absolute levels were diminished at the highest multiplicity of infection (MOI) tested (MOI of 750) in comparison to at lower

doses. Neither of the doses of heat-killed *B. pertussis* tested was found to be cytotoxic to the THP-1 cells based on trypan blue exclusion (data not shown). Overall, our data demonstrate that in heat-killed *B. pertussis*, the presence of GlcN substituents on lipid A leads to increased production of proinflammatory cytokines from human THP-1-derived M ϕ .

Lack of GlcN modification of the *B. pertussis* lipid A phosphate groups leads to diminished NF- κ B activation upon engagement with the human but not the murine TLR4-MD2-CD14 receptor complex. To confirm whether the differences in

FIG. 2. Secreted cytokines upon stimulation of THP-1-derived M ϕ with different doses of heat-killed whole cells of *B. pertussis* wild-type strain BP338 and its isogenic mutant BP338GlcN⁻. (A) Pyrogenic cytokines IL-1 β , IL-6, and TNF- α . (B) Chemokines IP-10, MCP-1, MIG, and RANTES. (C) MIP-1 α and MIP-1 β . Secreted levels of MIP-1 α and MIP-1 β after 24 h of induction with heat-killed *B. pertussis* BP338 exceeded the detectable range of the assay and therefore are not shown. (D) IFN- α and IFN- γ . Values shown are means $\pm$ standard errors of the means (SEM) from an experiment performed in triplicate. *P* values shown in the upper left corner of each graph were determined by two-way ANOVA with repeated measures. n.s., not significant; #, means from data points that were extrapolated beyond the highest standard concentration.

the abilities of heat-killed *B. pertussis* wild-type strain BP338 and mutant BP338GlcN⁻ to stimulate the release of inflammatory cytokines are indeed due to differential TLR4-mediated signaling, we used these bacteria to stimulate HEK-293 cells that were transiently transfected with cDNAs of TLR4, MD-2, and CD14 of either human or murine origin and an NF- κ B-dependent luciferase reporter construct. Unlike THP-1 cells, HEK-293 cells naturally lack the expression of the corresponding TLRs but can be transiently transfected to assess signaling through individual TLR receptors by quantifying the expression of the NF- κ B-dependent luciferase reporter. As shown in Fig. 3, heat-killed BP338GlcN⁻ bacteria were less potent inducers of signaling via the human TLR4–MD-2–CD14 receptor complex than the parental strain BP338. Surprisingly, the heat-killed bacteria from both strains were found to be equally potent agonists of the murine TLR4–MD-2–CD14 receptor complex, inducing a robust NF- κ B response in both cases. In transfected HEK-293 cells that expressed a hybrid receptor complex composed of either human MD-2, murine TLR4, and human CD14 or human TLR4, murine MD-2, and human CD14, the amount of NF- κ B activation upon stimulation with heat-killed cells of mutant BP338GlcN⁻ was again lower than what was observed upon stimulation with heat-killed cells of parental strain BP338, and it generally appeared to be less efficient than in HEK-293 cells expressing TLR4–MD-2–CD14 receptor complexes of solely human or murine origin. We also assessed the capacities of purified LOS from both strains to activate NF- κ B in HEK-293 cells that expressed either the human or the murine TLR4–MD-2–CD14 receptor complexes and obtained results similar to those shown in Fig. 3 (data not shown).

Lack of lipid A modification abolishes the ability of highly purified *B. pertussis* endotoxin to induce the release of inflammatory cytokines by human THP-1-derived macrophages but leads to only slightly reduced inflammatory cytokine levels when stimulating murine (RAW 264.7) macrophages. The NF- κ B activation experiments indicated that the human TLR4–MD-2–CD14 receptor complex is highly discriminatory between the wild-type and mutant strains that have or lacked the GlcN lipid A modification, whereas murine TLR4–MD-2–CD14 is not. To further verify these observations, we determined the abilities of purified LOS from both strains to stimulate the release of proinflammatory cytokines from either human THP-1-derived or murine RAW 264.7 M ϕ . Doses of LOS ranging from 1 ng/ml to 100 ng/ml were used, and cytokine levels were measured after stimulation for 4 and 24 h. We found that with THP-1-derived M ϕ , purified LOS from *B. pertussis* BP338 bearing the GlcN substituents was a potent inducer of the release of the pyrogenic cytokines IL-1 β , IL-6, TNF- α , and IFN- γ in a dose-dependent manner at both time points tested, whereas purified LOS from mutant BP338GlcN⁻ did not induce cytokine release at any dose and either time point tested (Fig. 4). With murine RAW 264.7 M ϕ , we found that purified LOS at doses of 10 ng/ml and 100 ng/ml from both the wild-type *B. pertussis* BP338 bearing the GlcN substituents and the mutant strain with free lipid A phosphate groups led to the release of IL-6 and TNF- α , albeit to slightly reduced levels in the latter case (Fig. 5). Interestingly, not only were the RAW 264.7 cells much less discriminatory between purified *B. pertussis* LOS with and without modified lipid A phosphate

FIG. 3. Relative light units as a readout of NF- κ B activation by transfected HEK-293 cells upon stimulation with different doses of heat-killed whole cells of *B. pertussis* wild-type strain BP338 and its isogenic mutant BP338GlcN⁻. HEK-293 cells were transiently transfected to express TLR4–MD-2–CD14 receptor complexes of either solely human (hu), solely murine (mu), or hybrid human and murine origin. Values represent means $\pm$ SEM from an experiment performed in triplicate. *P* values shown in the upper left corner of each graph were determined by two-way ANOVA with repeated measures. n.s., not significant; RLU, relative light units (firefly luciferase/*Renilla* luciferase).

FIG. 4. Secreted cytokines IL-1 β , IL-6, TNF- α , and IFN- γ upon stimulation of human THP-1-derived M ϕ with different doses of purified LOS of *B. pertussis* wild-type strain BP338 and its isogenic mutant BP338GlcN $^{-}$. Values shown are means $\pm$ SEM from an experiment performed in triplicate and are representative of two independent experiments performed in triplicate ($P < 0.0001$ for comparison of all data groups by regular two-way ANOVA). Linear regression analysis of all cytokine levels upon stimulation with purified LOS of the mutant showed no significance of the slope being different from zero, which is indicative of the lack of a cytokine response above background levels.

groups, but these cells were also less sensitive in detecting *B. pertussis* LOS, as no cytokine induction was detectable with a dose of 1 ng/ml at both time points tested.

DISCUSSION

In the present study we demonstrate that purified LOS and heat-killed whole bacteria of the *B. pertussis* Tohama I derivative BP338 elicit a pronounced increased release of a number

FIG. 5. Secreted cytokines IL-6 and TNF- α upon stimulation of murine RAW 264.7 M ϕ with different doses of purified LOS of *B. pertussis* wild-type strain BP338 and its isogenic mutant BP338GlcN $^{-}$. Values shown are means $\pm$ SEM from an experiment performed in triplicate and are representative of two independent experiments performed in triplicate. P values shown in the upper left corner of each graph were determined by two-way ANOVA with repeated measures.

of proinflammatory cytokines and chemokines by human THP-1-derived M ϕ when the lipid A phosphate groups are substituted with GlcN. Similar observations were made by Geurtsen et al. (14), who demonstrated a significant increase in the release of IL-6 by the human monocyte cell line MM6 when it was stimulated with killed whole-cell suspensions or purified LOS from *B. parapertussis* BPP535 bearing GlcN substituents at the lipid A phosphate groups, in comparison to an isogenic mutant that lacked this modification (14). They also observed a stimulatory affect of the GlcN modification in *B. pertussis*. However, IL-6 induction by their wild-type Tohama I derivative B213 was only modest (14). This was attributed to the low percentage of GlcN modification seen in this wild-type *B. pertussis* strain. In contrast to these findings, we have shown that purified endotoxin and heat-killed whole bacteria of wild-type *B. pertussis* can indeed elicit a robust cytokine response in human M ϕ . Accordingly, our MALDI MS spectra of LOS and lipid A of *B. pertussis* BP338 indicate a higher degree of GlcN modification than what was observed with *B. pertussis* B213. Since strains BP338 and B213 were both derived from *B. pertussis* Tohama I, the varying immunostimulatory activities of purified LOS and heat-killed whole-cell preparations of *B. pertussis* observed were likely due to differences in bacterial growth conditions. In fact, we have previously shown that the underlying gene locus is expressed differently upon cultivation of the bacteria on BG agar versus in SS broth (30), both standard media for the growth of *B. pertussis*. A minor residual peak was observed in the MS spectra of lipid A and highly purified LOS from *B. pertussis* mutant BP338GlcN $^{-}$ at m/z 1720. This mutant strain presumably displays some residual capacity to modify its lipid A, possibly due to the transcription

of the disrupted BP0398 locus and expression of truncated gene products with residual enzymatic activity. Nevertheless, the stimulations of human THP-1-derived M ϕ showed that purified LOS from this strain largely lacks significant immunostimulatory activity.

In support of the cytokine and chemokine data presented, we show that in comparison to purified LOS and heat-killed whole bacterial preparations of *B. pertussis* bearing the glucosamine substituents, those with free lipid A phosphate groups were less potent in their capacity to induce NF- κ B activation in transiently transfected HEK-293 cells that expressed the human TLR4-MD-2-CD14 receptor complex. This suggests that *B. pertussis* LOS were less potent agonists of the human TLR4-MD-2 receptor complex when the lipid A phosphate groups were not modified. This also explains the altered secretion levels of proinflammatory cytokines released by THP-1-derived M ϕ , since endotoxin-induced release of several of the cytokines tested here is dependent largely on signaling events that involve the Toll/IL-1 receptor (IL-1R) adapter protein MyD88 and the IL-1R-associated kinase IRAK-4 (25, 38, 51). Interestingly, Fedele et al. (13) have shown that purified *B. pertussis* BP338 LOS does not induce MyD88-independent signal transduction in human monocyte-derived dendritic cells (MDDC), and we have made similar observations with respect to the secretion of IFN- β from THP-1-derived M ϕ (N. Marr et al., unpublished data). Secretion of IP-10 in response to heat-killed *B. pertussis* BP338 cells was unexpected, because Fedele et al. (13) have reported that this chemokine was not further induced when MDDC were stimulated with purified *B. pertussis* BP338 LOS. Thus far, the regulation of IP-10 gene expression remains elusive, since it has been reported to require the activation of both the MyD88-independent interferon regulatory factor 3 (40) and the MyD88-dependent mitogen-activated protein (MAP) kinase signaling cascades (43). Again, our findings with respect to IP-10 may be a consequence of using different bacterial growth conditions than Fedele et al., which affected the degree of lipid A modification. Another possible explanation may be the induction of different cytokine/chemokine profiles in MDDC- versus THP-1-derived M ϕ .

The molecular mechanisms of the TLR4-MD-2-CD14 receptor which provide ligand specificity and enable the differentiation between various lipid A species have yet to be fully elucidated. Crystallographic analysis of the human TLR4 extracellular domain with and without bound MD-2 and of the tetra-acylated lipid A analogs lipid IVa and Eritoran have shown that when bound, all four acyl chains become virtually enclosed in a deep hydrophobic pocket of MD-2, leaving only the diglucosamine moiety exposed so that the distal phosphate groups can interact with the convex surface of the TLR4 extracellular domain which binds MD-2 (24, 36). Surprisingly, the crystal structure of the complex of human TLR4, MD-2, and hexa-acylated endotoxin of a rough-type *E. coli* strain revealed that the hydrophobic pocket of MD-2 accommodates only five of the six lipid A acyl chains, leaving the acyl chain in the C-2 position of the diglucosamine backbone to interact with TLR4. Moreover, the diglucosamine backbone of the *E. coli* lipid A moiety is rotated by ~ 180 degrees and displaced 4.5 to 5.5 Å outside the pocket compared to what was seen with the lipid A analogs lipid IVa and Eritoran (37). In this conformation, the lipid A phosphate groups are able to form ionic

interactions with both TLR4 and MD-2, thus contributing to the formation of two symmetrically arranged TLR4-MD-2 heterodimers, which in turn promotes signal activation through recruitment of intracellular adapter proteins such as MyD88 (37). Given the significance of the role of the lipid A phosphate groups during receptor multimerization, it will be important to elucidate the effect of the positively charged glucosamine substituents of the penta-acylated *B. pertussis* lipid A moiety on the receptor-ligand interaction.

Interestingly, regardless of whether the glucosamine substituents were present, purified LOS and heat-killed whole bacteria derived from *B. pertussis* strain Tohama I were equally potent NF- κ B activators in transfected HEK-293 cells when signaling proceeded through the murine TLR4-MD-2-CD14 receptor complex. Additionally, lack of the glucosamine substituents led to only a slight (but significant) reduction in the ability of purified LOS to induce the secretion of inflammatory cytokines in RAW 264.7 M ϕ . Consistent with our findings, *B. pertussis* has been shown elsewhere (19, 27) to stimulate a robust TLR4 response in mice or mouse-derived cells. Moreover, we found that HEK-293 cells that expressed hybrid TLR4-MD-2-CD14 receptor complexes comprised of human CD14 plus either murine TLR4 and human MD-2 or vice versa were more responsive to heat-killed *B. pertussis* with modified lipid A phosphate groups than those with free phosphate groups, although the responsiveness of the hybrid receptor complexes was generally impaired. This suggests that upon receptor-ligand interaction, human TLR4 and possibly human MD-2 are more discriminatory than their murine counterparts. Similar host-dependent ligand specificities of the TLR4-MD-2-CD14 receptor complexes have been reported previously (17). Whereas the murine TLR4-MD-2-CD14 receptor complex is activated in response to structurally different forms of LPS from *Pseudomonas aeruginosa*, such as a penta-acylated species that is abundant among environmental isolates as well as a hexa-acylated species with modified phosphate groups that is specific to isolates from airways of cystic fibrosis-affected individuals (12), robust activation of the human receptor complex is achieved only by the hexa-acylated LPS species (17). Similarly, lipid IVa lacks agonistic activity with respect to the human, but not the murine TLR4-MD-2-CD14 receptor complex (17). Host-dependent ligand specificities have also been reported for another PRR, the cytosolic Nod1 protein. Nod1 of murine origin mediates a strong response to tracheal cytotoxin, a peptidoglycan breakdown product released by viable cells of *B. pertussis*, whereas its human ortholog does not (26). Thus, different ligand specificities of the human and murine PRRs should be taken into account when interpreting previous observations made in mouse infection models, in which TLR4-mediated signaling has been indicated to play a role in clearance of the bacteria (20) and the generation of protective immunity from a whole-cell pertussis vaccine (19).

Our findings, which demonstrate that modification of the *B. pertussis* lipid A phosphate groups promoted rather than diminished signaling through the human TLR4-MD-2-CD14 receptor complex, were unexpected since expression of the genes responsible for the glucosamine modification is positively regulated by BvgAS (9, 30) and is therefore induced during the virulent phase of the bacterial infection. Given the central role of the BvgAS system in pathogenesis, we speculate

that the ability of *B. pertussis* to modify its lipid A has evolved to benefit the bacteria during human infection, possibly by modifying adaptive immune defense mechanisms, rather than to evade innate immune recognition; this requires further investigation. Modification of the lipid A phosphate groups of several Gram-negative bacteria, including *Salmonella enterica* serovar Typhimurium, *Pseudomonas aeruginosa*, and *Yersinia pseudotuberculosis*, with aminoarabinose confers resistance to cationic antimicrobial peptides such as polymyxin B (15, 28, 34, 49). However, we have found that *B. pertussis* strain BP338 is considerably sensitive to polymyxin B, with the MICs for both wild-type BP338 and the isogenic transposon mutant BPM2859 with free lipid A phosphate groups being 0.05 µg/ml (29).

Finally, whether or not *B. pertussis* promotes a TLR4-mediated inflammatory response may also have implications for the formulation of whole-cell pertussis component vaccines. Due to their low costs, these vaccines are still being used to immunize children in developing countries despite their higher reactogenicity in comparison to the generally equally efficacious but significantly more expensive acellular pertussis vaccines that contain selected purified *B. pertussis* protein antigens instead of whole killed bacteria. Earlier studies have reported a significant positive association of endotoxin units measured by the *Limulus* amoebocyte lysate assay and the percentage of vaccine recipients who developed fever (3). The use of genetically manipulated *B. pertussis* strains that lack the ability to modify their lipid A may therefore be superior to the use of strains such as Tohama I for vaccine formulation if they elicit less frequent or diminished side effects such as local inflammation or fever.

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