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Role of D-Alanylation of *Streptococcus gordonii* Lipoteichoic Acid in Innate and Adaptive Immunity[▽]

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In recent years, there has been considerable interest in using the oral commensal gram-positive bacterium *Streptococcus gordonii* as a live vaccine vector. The present study investigated the role of D-alanylation of lipoteichoic acid (LTA) in the interaction of *S. gordonii* with the host innate and adaptive immune responses. A mutant strain defective in D-alanylation was generated by inactivation of the *dltA* gene in a recombinant strain of *S. gordonii* (PM14) expressing a fragment of the S1 subunit of pertussis toxin. The mutant strain was found to be more susceptible to killing by polymyxin B, nisin, magainin II, and human β defensins than the parent strain. When it was examined for binding to murine bone marrow-derived dendritic cells (DCs), the *dltA* mutant exhibited 200- to 400-fold less binding than the parent but similar levels of binding were shown for Toll-like receptor 2 (TLR2) knockout DCs and HEp-2 cells. In a mouse oral colonization study, the mutant showed a colonization ability similar to that of the parent and was not able to induce a significant immune response. The mutant induced significantly less interleukin 12p70 (IL-12p70) and IL-10 than the parent from DCs. LTA purified from the bacteria induced tumor necrosis factor-alpha and IL-6 production from wild-type DCs but not from TLR2 knockout DCs, and the mutant LTA induced a significantly smaller amount of these two cytokines. These results show that D-alanylation of LTA in *S. gordonii* plays a role in the interaction with the host immune system by contributing to the relative resistance to host defense peptides and by modulating cytokine production by DCs.

Lipoteichoic acid (LTA) is an amphiphilic polymer of polyphosphoglycerol or polyphosphoribitol, anchored to the cytoplasmic membrane by a glycolipid. Each phosphoglycerol or phosphoribitol on this molecule may be modified with glycosylation or, more often, may contain D-alanine (10, 11). The *dlt* operon, which contains four genes, *dltA*, *dltB*, *dltC*, and *dltD*, is responsible for the D-alanylation of teichoic acid and lipoteichoic acid. The *dltA* gene encodes the D-alanyl carrier protein ligase which activates the D-alanine for ligation to the D-alanyl carrier protein (DltC). Disruption of any one of the *dlt* genes will eliminate D-alanylation (30). D-Alanylation confers a positive charge to the LTA and has been demonstrated to have a role in the regulation of autolytic activity (12) as well as a role in binding Mg^{2+} which is essential for the activation of Mg^{2+}-dependent membrane enzymes (2).

A number of studies have demonstrated that D-alanylation in *Streptococcus agalactiae*, *Staphylococcus aureus*, and group A *Streptococcus* spp. plays a role in escaping host defense peptides (HDP) (19, 32, 33, 41). HDP are recognized as one of the critical components of the innate immune system. These amphipathic peptides target and exert antimicrobial activity on cells with a negatively charged surface by disrupting transmembrane potential and lipid symmetry, which results in cell lysis (13). Studies have also shown that the lack of D-alanylation leads to increased susceptibility to phagocytic cells (5, 33), decreased adherence to macrophages (1), loss of ability to colonize cotton rat nares (41), and increased susceptibility to neutrophil killing (20). Since D-alanylation appears to have a role in the interaction of gram-positive bacteria with the host immune system, it would stand to reason that D-alanylation may have a role in modulating the immune response.

Streptococcus gordonii is a commensal gram-positive organism that naturally resides in the human oral mucosa. In recent years, this organism has been under study as a potential vector for live oral vaccines (23). *S. gordonii* is a favorable vaccine vector due to its nonpathogenic nature, early colonization of the oral cavity, persistence in colonization, and ability to express heterologous antigens on its surface. *S. gordonii* has recently been used in a phase I clinical trial to demonstrate that the organism can be safely administered to humans by the nasal/oral route and colonization can be quickly eliminated by antibiotic treatment (18). However, eliciting a strong immune response with recombinant *S. gordonii* has been difficult. In previous studies, the oral colonization of mice with recombinant *S. gordonii* PM14 surface expressing the S1 fragment of pertussis toxin elicited only a weak mucosal immune response (24). Since eliciting a strong immune response is critical to the use of *S gordonii* as an oral vector, it is important to understand how *S. gordonii* interacts with the components of the innate and adaptive immune systems and what role D-alanylation may play in that interaction. A previous study by Clemans et al. (4) created a *dltA* mutant from *S. gordonii* DL1 (Challis), but this group only explored the effects of the mutation on intragenic coaggregations.

In this study, we insertionally inactivated the *dltA* gene in *S.*

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant property	Reference or source
Strains		
S. gordonii		
DL1	Wild type	D. LeBlanc
PM14	DL1 harboring pPM14	27
PM14 *dltA*	*dltA* mutant of PM14, gene insertionally inactivated with *ermAM*	This study
E. coli PK3330	DH5α containing pDC11	4
S. pyogenes	Type M22	Claes Schalen, Lund University, Sweden
Plasmids		
pDC11	pBluescript KS(+) carrying the *S. gordonii* DL1 *dltA* gene interrupted with *ermAM*	4
pPM14	*xyl tet* promoter expressing the S1 subunit of pertussis toxin, pDL276 backbone (13 kb), Kan^r	27

gordonii to determine the role of D-alanylation in modulating immune responses. More specifically, we examined the role of *S. gordonii* lipoteichoic acid (LTA) D-alanylation in cationic antimicrobial peptide resistance, the interaction with dendritic cells (DCs), and the induction of cytokines from DCs. The interactions of *S. gordonii* with DCs are explored because these immune cells have an important role in dictating the subsequent adaptive immune response (25).

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study are listed in Table 1. Streptococci were cultured in a brain heart infusion (BHI; Becton, Dickinson and Company, Sparks, MD) broth at 37°C aerobically without shaking and on BHI agar or Todd-Hewitt agar (Becton Dickinson and Company) in candle jars. *S. gordonii* strains used for the MIC assays were grown in Mueller-Hinton broth (Becton Dickinson and Company) supplemented with 3% (wt/vol) glucose. Antibiotics, when needed, were included in the media at 10 μg/ml erythromycin or 250 μg/ml kanamycin. *Escherichia coli* PK3330 was cultured aerobically at 37°C in Luria-Bertani broth (1% [wt/vol] Bio-tryptone, 1% [wt/vol] NaCl, and 0.5% [wt/vol] yeast extract) with 100 μg/ml ampicillin and 300 μg/ml erythromycin.

Insertional inactivation of the *S. gordonii dltA* gene with *ermAM*. To inactivate the *dltA* gene in *S. gordonii*, the previously described pDC11 carrying the *dltA*::*ermAM* construct was used (4). pDC11 is a pBluescript derivative with a 1.4-kb BamHI-KpnI fragment of the *S. gordonii* DL1 *dltA* gene insertionally inactivated by *ermAM* (922 bp). The pDC11 plasmid was isolated from *E. coli* by using an alkaline lysis method (3). The plasmid was subsequently linearized using BamHI and used in the natural transformation of *S. gordonii* PM14 by using methods previously described (21). Transformants were selected on BHI agar containing kanamycin and erythromycin.

Genomic DNA was isolated from the transformants and used as templates for amplification of the interrupted *dltA* gene by PCR. A typical PCR consisted of 1 μl of a 1/100 dilution of *S. gordonii* genomic DNA, 50 pmol of each of the primers SL355 (CCGGATCCTGACCTCGCTGATTAAGCCC) and SL356 (GGGGGTACCTCTCCTGTCGTGGTCTATGGTGGGC), 2 mM $MgCl_2$, and 2.5 U *Taq* DNA polymerase (Invitrogen Life Technologies, Burlington, ON) in a final reaction mixture volume of 100 μl. PCR was carried out under the following conditions: 2 min at 94°C; 30 cycles of 1 min at 94°C, 30 s at 54°C, and 2.5 min at 72°C.

Isolation of LTA. LTA was isolated from *S. gordonii* by using the hot phenol extraction method (32). Cells from a 500-ml overnight culture were harvested by centrifugation and washed in cold sodium acetate buffer (0.1 M, pH 4.7). Cells were resuspended in the same buffer and disrupted using glass beads in a cell disintegrator (Mickle Laboratory Engineering Co. Ltd., Gonshall, Surrey, United Kingdom) for 1 h at 4°C. The crude cell extract was recovered and centrifuged for 15 min at 300 × *g*. The supernatant was mixed with an equal volume of prewarmed 80% (wt/vol) aqueous phenol and stirred at 65°C for 1 h. The solution was cooled at room temperature for 10 min and centrifuged for 30 min at 300 × *g*, and the aqueous layer was collected. An equal volume of sodium acetate buffer was then added to the phenol layer, stirred again at 65°C for 1 h, cooled, and centrifuged. Once again, the aqueous layer was collected. The two aqueous fractions were combined and dialyzed against 0.1 M sodium acetate buffer (pH 5.0). The LTA extracts were subsequently freeze-dried.

For the isolation of LTA to be used in dendritic cell stimulation assays, *S. gordonii* was grown in 500 ml of BHI prepared in endotoxin-free water (Invitrogen). The cells were disrupted as described above, and LTA was isolated by butanol extraction, followed by hydrophobic interaction chromatography on octyl-Sepharose as described by Morath et al. (29). In the isolation process, care was taken to prevent the introduction of endotoxin by ensuring reagents were prepared in endotoxin-free water and containers. The purified LTA was analyzed on a 7.5% polyacrylamide gel in Tris-borate-EDTA buffer (42) and detected as a single band by the cationic dye Stains-All (Sigma-Aldrich) (22). The purified LTA was then freeze-dried.

Analysis of D-alanine and phosphorus contents. The amounts of D-alanine in the LTA samples were determined using the method described by Peschel et al. (32). LTA samples (5 mg) were adjusted to a pH of 9 to 10 with NaOH to a final volume of 100 μl and were incubated for 1 h at 37°C to hydrolyze the D-alanine esters. Samples were then incubated for another hour at 37°C following the addition of 200 μl of 0.2 M Tris-HCl (pH 8.4) containing 8.5 U of amino acid oxidase (Sigma-Aldrich Chemical Co., Oakville, ON) to convert D-alanine to pyruvate. Trichloroacetic acid (30%; 100 μl) was added to inactivate the oxidase enzyme. Finally, 100 μl of 2,4-dinitrophenylhydrazine (0.1% solution in 2 M HCl) was added to the solution and incubated for 5 min at room temperature. The reaction was stopped with 200 μl of 2.5 M NaOH and the absorbance at 525 nm was determined. A standard curve was constructed using D-alanine (Sigma-Aldrich).

The amount of phosphorus in the LTA samples was determined according to the method described by Zhou and Arthur (43). Briefly, the LTA samples (50 μg/μl) were boiled in 2 M HCl for 2.5 h. Ten microliters of 70% $HClO_4$ was added to various amounts of the LTA samples, and distilled water was added to make a total volume of 200 μl. Finally, 1 ml of malachite green solution (3 volumes of 0.4% [wt/vol] malachite green in distilled water, 1 volume of 4.2% ammonium molybdate in 5 M HCl, 0.06% Tween 20) was added, and the solution was allowed to stand for 20 min before the absorbance at 660 nm (A_{660}) was determined. A standard curve was constructed using K_2PO_4.

Alcian blue binding assay. *S. gordonii* cells grown in BHI were harvested at mid-exponential phase (optical density at 600 nm [OD_{600}] of 0.5) and were washed once by centrifugation (5 min at 10,000 × *g*) with 20 mM morpholinepropanesulfonic acid (MOPS) buffer (pH 7). The cells were resuspended in MOPS buffer to a final OD_{600} of 0.5, and the cationic dye Alcian blue 8GX (Sigma-Aldrich) was added to a final concentration of 65 μg/ml. Samples were rotated at 3 rpm at room temperature. After 10 min, the mixtures were centrifuged to pellet the bacteria. The unbound Alcian blue in the supernatant fluids was measured at 650 nm using a spectrophotometer. In parallel, tubes containing the same amount of Alcian blue in MOPS buffer but without bacteria were treated similarly, as controls. The amount of Alcian blue bound to the bacteria was calculated as (A_{530} of supernatant without bacteria − A_{530} of supernatant with bacteria)/A_{530} of supernatant without bacteria × 100.

MIC assays. The MIC of polymyxin B sulfate (Fluka, Buchs, Switzerland) was determined in 96-well polypropylene microplates (Corning Inc., Corning, NY) by a microdilution method. Bacteria (50 μl; 10^7 CFU) were added to the wells, which contained 50 μl of twofold-diluted polymyxin B. The starting concentra-

tion for polymyxin B was 200 μg/ml. Plates were incubated at 37°C for 24 h and examined visually for bacterial growth. The lowest concentration that inhibited growth was considered to be the MIC.

Susceptibility to cationic antimicrobial peptides in kinetic inhibition assays. These assays were performed in Mueller-Hinton broth supplemented with glucose in 1.5-ml Eppendorf tubes. Bacteria (10^7 CFU in 100 μl) were added to tubes containing 100 μl of one concentration of polymyxin B sulfate, nisin, magainin II, human β defensins 1 and 2, or histatin 5, and these samples were incubated at 37°C. All peptides were used at a concentration of 200 μg/ml, with the exception of that of magainin II, which was 225 μg/ml. Survival over time was determined by plating on Todd-Hewitt agar in triplicate and counting the resulting colonies after incubation for 48 h. All peptides except for human β defensins 1 and 2 were purchased from Sigma-Aldrich. Human β defensins 1 and 2 were purchased from Peptides International Inc. (Louisville, KY).

DC culture. Bone marrow-derived DCs were cultured, using the method described by Lutz et al. (26), from female BALB/c mice (Charles River Laboratories, St. Constant, PQ) or from female Toll-like receptor 2 (TLR2) knockout mice. Briefly, on day 0, femurs and tibia of the mice were flushed, and the resulting bone marrow suspension was passed through a 70- or 100-μm-pore-size cell strainer (BD Biosciences, Mississauga, ON) to obtain a single-cell suspension. Red blood cells were subsequently lysed using a hypotonic 0.2% (wt/vol) NaCl solution, and a hypertonic 1.6% (wt/vol) NaCl solution was added to restore equilibrium. The cells were then seeded at 0.2 million per ml of RPMI medium with L-glutamine (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum, 2% HEPES, 1% penicillin-streptomycin, 0.003% β-mercaptoethanol, and 20 or 40 ng/ml of murine granulocyte macrophage colony-stimulating factor (rGM-CSF; Peprotech Inc., Rocky Hill, NJ). Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. On day 3, fresh RPMI medium containing rGM-CSF was added to double the original volume. On day 6, the nonadherent cells were harvested and used for subsequent experiments.

Cell binding assays. An in vitro fluid-phase assay was used to investigate the binding between *S. gordonii* and murine DCs or HEp-2 cells. Mouse DCs were cultured as described above. HEp-2 cells (ATCC accession number CCL-23; Manassas, VA) were grown to a confluent monolayer, and cells were detached using HyQtase trypsin replacement (Sigma-Aldrich). To prevent phagocytosis by the DCs, all reagents were chilled to 4°C prior to use, and the incubation was performed at 4°C. Early exponential-phase *S. gordonii* cells were washed once with phosphate-buffered saline (PBS) and resuspended in PBS. The mammalian cells were also washed and resuspended in PBS. Cells and bacteria were mixed in a ratio of 1:50, using 1 million DCs or HEp-2 cells per sample, and rotated at 4 rpm. Samples were taken at 20, 30, and 45 min and centrifuged at $100 \times g$ for 3 min. The pellet was washed once with BHI and resuspended in 50 μl of BHI. The pellet was sonicated for 5 seconds at an amplitude of 10 (Vibracell Sonics & Materials Inc., Danbury, CT) to lyse the mammalian cells but not the bacteria. The sample was then serially diluted and plated in triplicate on selective agar. All plates were then incubated for 48 h, and colonies were counted.

Mouse colonization study. An animal trial with mice was conducted to determine if the *S. gordonii dltA* mutant could colonize the oral mucosa of mice and to determine the immune response to this recombinant bacterium. Five-week-old female BALB/c mice ($n = 5$) were given two consecutive intraoral/intranasal doses of 10^9 CFU of the parent or mutant *S. gordonii* on days 1 and 2, using methods described previously (24). Oral swabs were taken on days 3, 10, 17, and 24. Saliva was collected on day 27, and sera were collected by heart puncture when mice were euthanized on day 28. At the time of euthanasia, swabs of the oral cavity, pharynx, trachea, and nasal cavity were taken. Swabs were placed in 1 ml of ice-cold BHI broth and vortexed for 1 min. Bacterial counts for *S. gordonii* were determined by plating the broth on BHI containing antibiotics and for total aerobes and facultative anaerobes on sheep blood agar. Sample colonies from the BHI plates were regrown in BHI broth, and the proteins were extracted with sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer (17). Western immunoblotting was performed with these samples to confirm the identity of the streptococci recovered from the oral cavity. The blots showed that the protein samples from the swab colonies were expressing the SpaPS1 recombinant protein, which was recognized by anti-S1 monoclonal antibody A4 (data not shown).

The generation of specific antibody responses in mice against pertussis toxin or *S. gordonii* was evaluated using enzyme-linked immunosorbent assays (ELISA) as described previously (24). Microtiter plate wells were coated with 100 ng of pertussis toxin (Chiron Inc., Emeryville, CA) or *S. gordonii* PM14 fixed with 0.125% glutaraldehyde. Anti-pertussis toxin and anti-*S. gordonii* immunoglobulin G (IgG) antibodies in sera were assayed with a starting dilution of 1/20. The IgG was detected with the alkaline phosphatase-conjugated goat anti-mouse IgG (1/7,500 dilution; Sigma-Aldrich). Anti-pertussis toxin and anti-*S. gordonii* IgA antibodies in saliva (1/20 dilution) were detected with biotin-conjugated goat anti-mouse IgA antibody (1/20,000 dilution; Sigma-Aldrich), followed by avidin-alkaline phosphatase conjugate (1/20,000 dilution; Sigma-Aldrich).

DC stimulation. An in vitro stimulation was used to investigate the induction of cytokines from DCs. Six-day-old mouse DCs were cultured as above and seeded at 1×10^6 cells/well in 24-well tissue culture plates. Late-exponential-phase wild-type cells, the *dltA* mutant, and *S. pyogenes* were grown in BHI. The medium was prepared with endotoxin-free water and sterilized through a 0.2 μM filter into endotoxin-free plastic conical tubes. The bacterial cultures were washed once with endotoxin-free, sterile PBS (Invitrogen) and added to DCs at three different ratios of bacteria to DCs (1:1, 2.5:1, and 5:1). DCs were also stimulated with purified LTA at the concentrations indicated. In all stimulation assays, DCs were stimulated with 1 μg/ml lipopolysaccharide (LPS; *E. coli*; Sigma-Aldrich) or left unstimulated for 24 h as controls. Each treatment was replicated in duplicate or triplicate.

Cytokine ELISAs. Cytokine concentrations in the DC culture supernatants were determined by ELISA using capture and detection antibodies and cytokine standards for mouse tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), IL-4, IL-12p70, IL-2 (R&D Systems Inc., Minneapolis, MN), and IL-10 (Peprotech, Rocky Hill, NJ). Maxisorp Nunc plates were coated with 50 μl of 1 μg/ml capture antibody, and the undiluted supernatants or the cytokine standards were added to the wells. Biotinylated detection antibody (50 μl of 0.2 μg/ml) was used to detect each cytokine, and an ELISA Amplification System (Invitrogen) was used according to the manufacturer's instructions to amplify the signal. The reaction was stopped by using 50 μl of 0.3 M H_2SO_4, and plates were read at 490 nm. Statistical significance of the results was evaluated by analysis of variance, followed by the Tukey test. A P value of less than 0.05 was considered to be significant.

RESULTS

Insertional inactivation of the *S. gordonii dlt* operon and analysis of the D-alanine content in LTA. To create a *dltA* mutant strain in *S. gordonii* PM14, the previously described *dltA*::*ermAM* construct (4) was introduced into the bacterium by transformation. Via allelic exchanges, the *ermAM* gene was inserted into the chromosome, resulting in the inactivation of *dltA*. The inactivation of the *dltA* gene was demonstrated by PCR using primers specific for the *dltA* gene that showed the amplification of the expected 2.3-kb DNA fragment from the mutant and the intact 1.4-kb *dltA* gene from the parent strain (data not shown). LTA samples from the parent strain and the *dltA* mutant were analyzed for D-alanine content. No detectable amount of D-alanine was found in the mutant LTA, while the parent LTA contained 3.27 μg of D-alanine per mg of LTA or 6.2 mol of D-alanine per mole of phosphorus. These results indicate that the *dlt* gene had been inactivated.

The *dltA* mutant and wild-type *S. gordonii* showed similar growth rates. The results from the cationic dye Alcian blue 8GX binding assay showed that the mutant had an increase in negative surface charges. The mutant bound 72.2% ± 1.64% of the Alcian blue in comparison to a 62.4% ± 1.67% binding by the wild type ($P < 0.001$).

Susceptibility to antimicrobial peptides. The MIC of polymyxin B was determined for the *S. gordonii* PM14 wild type and the *dltA* mutant. The mutant was markedly more sensitive than the parent strain to polymyxin B. The MIC of polymyxin B displayed by the parent strain was 200 μg/ml, while that by the mutant was 3.125 μg/ml.

The susceptibility to antimicrobial peptides was further investigated in a kinetic inhibition assay. As shown in Fig. 1, the mutant was highly susceptible to inhibition by polymyxin B, nisin, and magainin II. The mutant was also susceptible to human β-defensins 1 and 2, while the parent was not inhibited

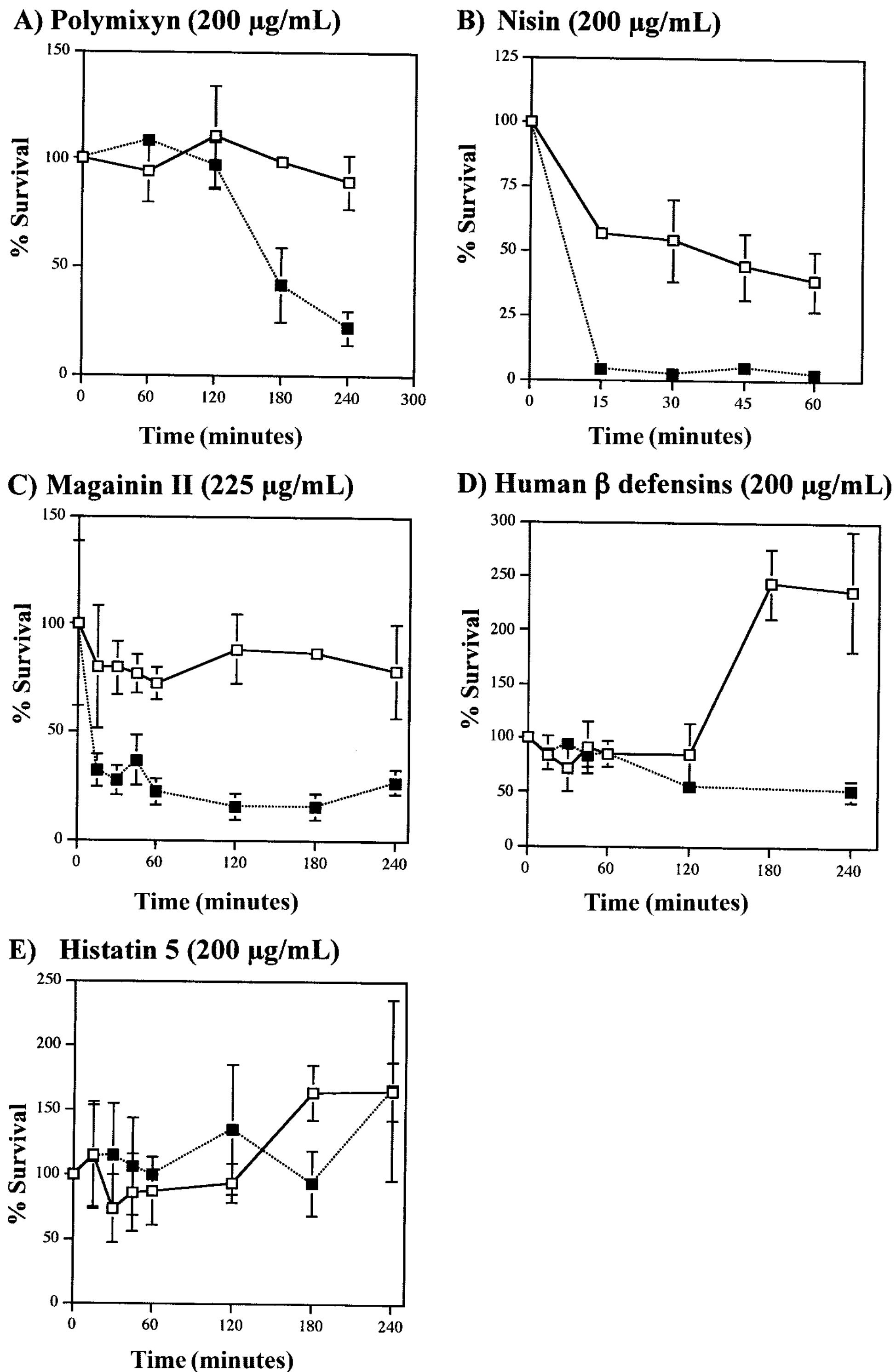

FIG. 1. (A to E) Susceptibilities of *S. gordonii* PM14 (open squares) and the *dltA* mutant (filled squares) to various antimicrobial peptides. Data for panels A, B, and E represent the means of two independent experiments. Data in panels C and D are means ± standard deviations of triplicates from a single experiment.

and continued to grow in the presence of these two peptides. Interestingly, the mutant and the parent strains were not susceptible to histatin 5, an antimicrobial peptide found in human saliva, with a net charge of +5.

Oral colonization in BALB/c mice. The ability of the *dltA* mutant to colonize orally was tested with BALB/c mice. As shown in Table 2, the *dltA* mutant and parent bacteria showed no differences in colonization. Swabs at the time of euthanasia

TABLE 2. Colonization of BALB/c mice by *S. gordonii* PM14 and the *dltA* mutant[a]

Site	PM14-inoculated mice (mean CFU ± SE)		DltA-inoculated mice (mean CFU ± SE)	
	Total aerobes and facultative anaerobes	*S. gordonii*	Total aerobes and facultative anaerobes	*S. gordonii*
Oral cavity	82,600 ± 40,060	657 ± 576 (0.8%)	34,063 ± 8,068	1,267 ± 381 (3.8%)
Pharynx	8,500 ± 2,540	133 ± 105 (1.7%)	5,753 ± 1,650	210 ± 80 (4.0%)
Trachea	1,135 ± 498	13 ± 12 (1.2%)	194 ± 76	13 ± 12 (6.8%)
Nasal cavity	70 ± 61	0 (0%)	1,480 ± 2,761	3 ± 7 (0.2%)

[a] The mean totals of CFU recovered from each site on the upper respiratory tract and oral cavity are shown along with the standard errors (SE) of the means ($n = 5$). The number in parentheses is the number of *S. gordonii* bacteria expressed as a percentage of the total aerobes and facultative anaerobes.

of the oral cavity, pharynx, trachea, and nasal cavity revealed similar levels of colonization at all of these sites between the parent and mutant strains. The oral cavity, pharynx, and trachea were colonized with a number of recombinant *S. gordonii* strains which represented a proportion of the anaerobes and facultative anaerobes normally found in these locations (0.8 to 6.8%). The nasal cavity was not well colonized by the either the parent or the mutant strain.

The production of anti-pertussis toxin and anti-*S. gordonii* serum IgG and salivary IgA antibodies following colonization was assessed by ELISA. IgG or IgA anti-pertussis and anti-*S. gordonii* antibodies were not found in sera and saliva samples from either group.

Binding of *S. gordonii* to mouse dendritic cells and HEp-2 cells. To determine if D-alanylation plays a role in binding to DCs, *S. gordonii* PM14 and the *dltA* mutant were incubated with immature bone marrow-derived DCs from wild-type and TLR2 knockout mice. These DC binding assays were carried out at 4°C to prevent phagocytosis. The parent *S. gordonii* strain bound 200- to 400-fold better to normal DCs than to cells of the *dltA* strain at all three time points (Fig. 2). An average of 1 to 3% (0.39×10^6 to 1×10^6 CFU/ml) of the parent bacteria added to an assay was cell bound, while the mutant exhibited an average of 0.01% (1.98×10^3 to 3.21×10^3 CFU/ml) binding to the normal DCs. The difference in the mean percentage of the parent versus that of mutant bacteria bound to normal DCs was statistically significant at the 30-min ($P = 0.05$) and 45-min ($P = 0.04$) time points.

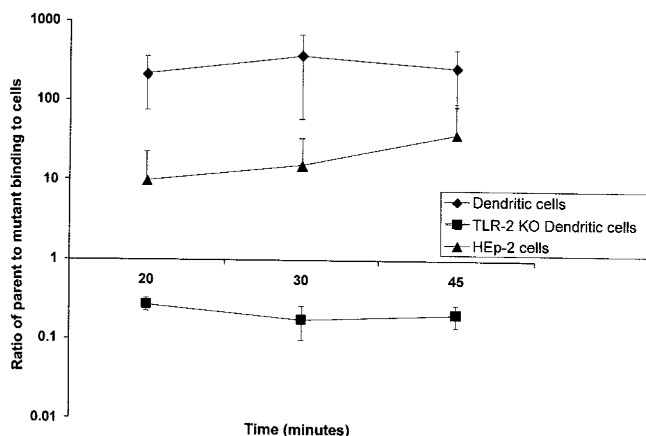

FIG. 2. Adherence of *S. gordonii* PM14 and the *dltA* mutant to bone marrow-derived dendritic cells from wild-type and TLR2 knockout (KO) mice and HEp-2 cells. Cells were cultured in vitro and incubated for 20, 30, or 45 min with early log-phase bacteria. Samples were run in triplicate, and the mean ratios of parent to mutant bacteria bound ± standard deviations of two or three independent experiments are shown.

Binding assays were also performed using TLR2 knockout DCs to investigate whether TLR2 was the receptor mediating this difference in binding between the parent and mutant *S. gordonii* to normal DCs. If the binding were mediated by TLR2, the lack of this receptor would impair the binding of the parent *S. gordonii* to the TLR2 knockout DCs. Surprisingly, the parent *S. gordonii* exhibited levels of binding to the TLR2 knockout DCs that were similar to those of the normal DCs, while the levels of *dltA* mutant binding to TLR2 knockout DCs increased (Fig. 2). The numbers of bound parent and mutant bacteria were 1.51×10^5 to 2.13×10^5 CFU/ml and 7.36×10^5 to 8.23×10^5 CFU/ml, respectively. The differences in binding levels between the parent and the mutant bacteria was not statistically significant at any of the time points.

D-Alanylation of LTA in *S. pyogenes* has been shown to play an important role in the attachment to human epithelial cells (19). In order to determine if the same was true for *S. gordonii*, the binding of the parent strain incubated with HEp-2 cells was compared to that of the *dltA* mutant incubated with HEp-2 cells. The results showed that the numbers of bound parent and mutant bacteria were 1.03×10^7 to 1.35×10^7 CFU/ml and 0.56×10^6 to 1.69×10^6 CFU/ml, respectively. Thus, there was generally more parent than mutant *S. gordonii* bound to the HEp-2 cells (~10-fold), but the difference was not statistically significant.

Induction of cytokines by *S. gordonii* from dendritic cells. To determine if D-alanylation of LTA in *S. gordonii* plays a role in modulating the cytokine response, DCs were stimulated with *S. gordonii*, and the amounts of two proinflammatory cytokines (TNF-α and IL-12), the Th2/regulatory cytokine IL-6, the regulatory cytokine IL-10, and the proinflammatory/regulatory cytokine IL-2 were assayed by ELISA. Both the parent and the mutant were able to induce higher levels of TNF-α, IL-12p70, IL-6, IL-10, and IL-2 than the unstimulated control (Fig. 3). As expected, DCs stimulated with LPS also produced these cytokines. The levels of IL-12p70 and IL-10 induced by the parent strain were significantly higher than that induced by the *dltA* mutant at all three ratios (Fig. 3A and B). Similar levels of TNF-α, IL-6, and IL-2 were induced by both *S. gordonii* strains (Fig. 3C to E). As a comparison, the cytokine profile induced by *S. pyogenes* was also examined. Like *S. gordonii*, *S. pyogenes* was capable of inducing higher levels of cytokines than the unstimulated control (Fig. 3). The level of IL-2 induced by *S.*

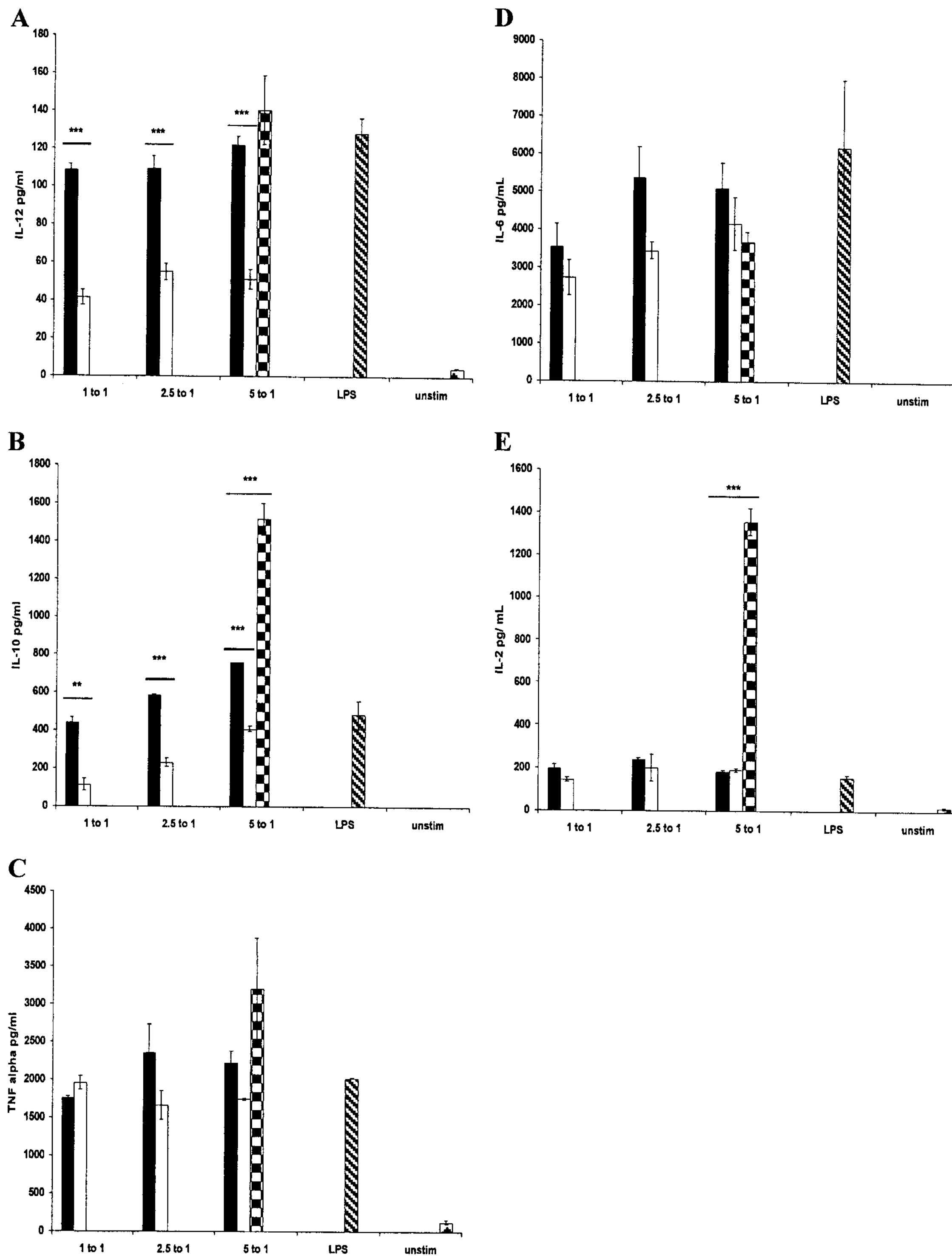

FIG. 3. (A to E) Cytokine production by 6-day-old bone marrow-derived dendritic cells from BALB/c mice in response to stimulation by *S. gordonii* (black bars), the *dltA* mutant (white bars), and *S. pyogenes* (square checkers), or LPS (hatched bars) or left unstimulated (unstim; diamond checkers). DCs were stimulated with *S. gordonii* PM14 and its *dltA* mutant at a bacterium:DC ratio as shown. Stimulation with *S. pyogenes* was performed only at the 5:1 ratio. ***, $P < 0.001$; **, $P < 0.01$.

pyogenes was markedly higher than that induced by *S. gordonii*. Similarly, the level of IL-10 induced by *S. pyogenes* was significantly higher than that induced by *S. gordonii*, while the TNF-α level induced by the former was higher but not statistically significant. The levels of IL-6 induced by *S. pyogenes* and *S. gordonii* were similar.

In order to determine whether TLR2 plays a role in mediating the cytokine profile induced by the *S. gordonii* strains,

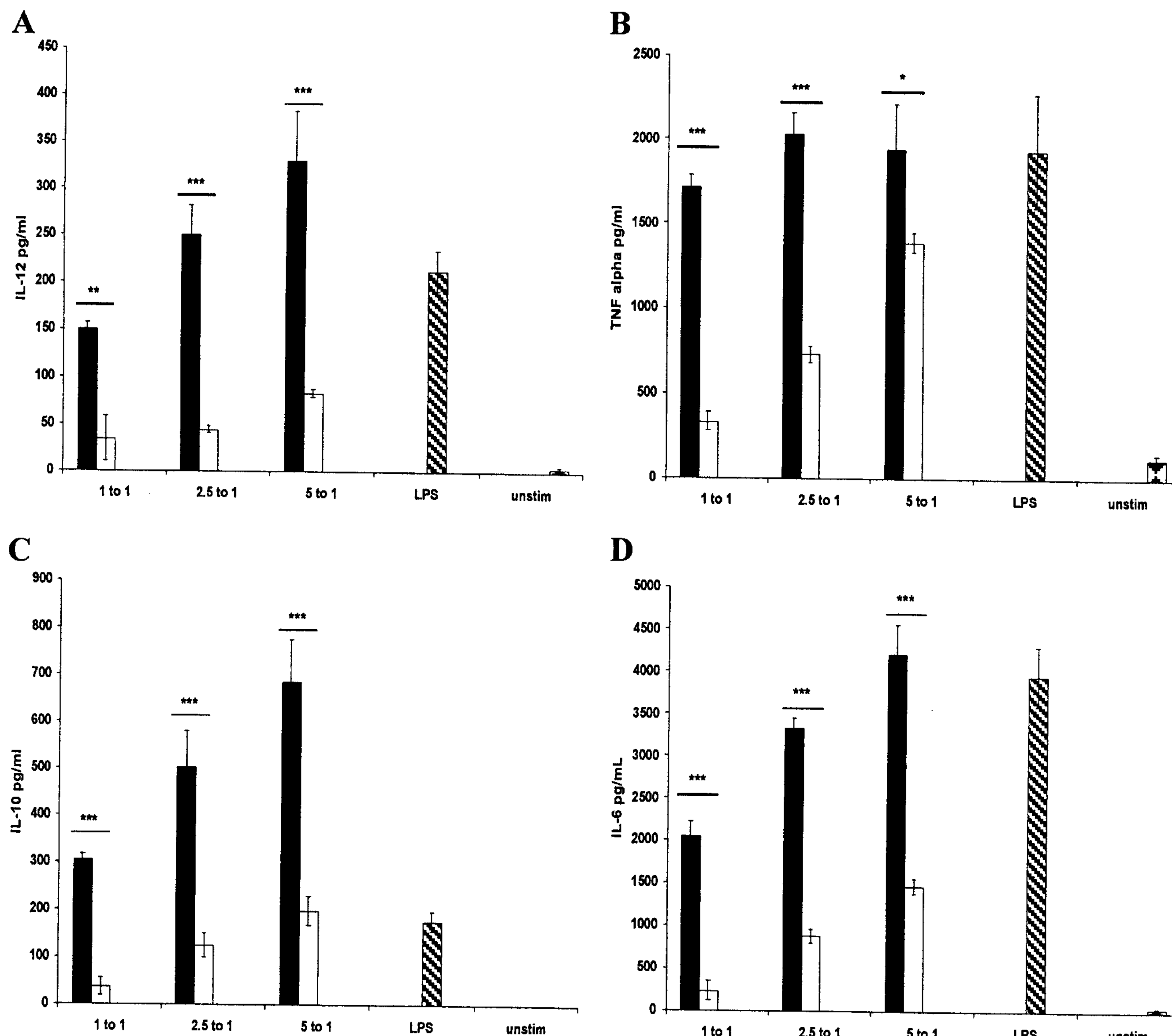

FIG. 4. (A to D) Cytokine production by 6-day-old bone marrow-derived dendritic cells from TLR2 knockout mice in response to stimulation by *S. gordonii* (black bars), the *dltA* mutant (white bars), or LPS (hatched bars) or left unstimulated (unstim; diamond checkers). DCs were stimulated with *S. gordonii* PM14 and its *dltA* mutant at the bacterium/DC ratios shown. Stimulation with *S. pyogenes* was not performed. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

TLR2 knockout DCs were used with the stimulation assays. The results showed that both the parent and the *dltA* strains were able to induce TNF-α, IL-12p70, IL-6, and IL-10 production from the DCs (Fig. 4). However, a clear difference between the abilities of the parent and the *dltA* mutant to induce cytokine production by the TLR2 knockout DCs was observed. The mutant induced significantly less production of all 4 of these cytokines than the parent. The levels of TNF-α, IL-6, and IL-10 produced by the TLR2-deficient DCs were similar to that produced by the normal DCs in response to parent *S. gordonii* stimulation. However, the level of IL-12p70 produced by the TLR2-deficient DCs was 1.5 to 3.5 times higher than that produced by the normal DCs. Hence, the difference in response exhibited by normal and that of TLR2 knockout DCs was apparently due largely to the decreased cytokine levels induced by the *dltA* mutant. The levels of IL-2 were undetectable in any of the samples, including the LPS and unstimulated controls. It was also observed that stimulation with the *S. gordonii* strains had a clear dose-dependent effect on the cytokines levels that were induced (TNF-α, IL-6, IL-10, and IL-12p70). When more bacteria were used to stimulate the DCs, higher levels of cytokines were produced.

Induction of cytokines by LTA. To further examine the role of D-alanylation of LTA in modulating cytokine response, DCs were stimulated with purified LTA. When wild-type DCs were stimulated with LTA, a dose-dependent production of TNF-α and IL-6 was observed, while no IL-12p70 or IL-10 was detected even when LTA was used at 400 μg/ml

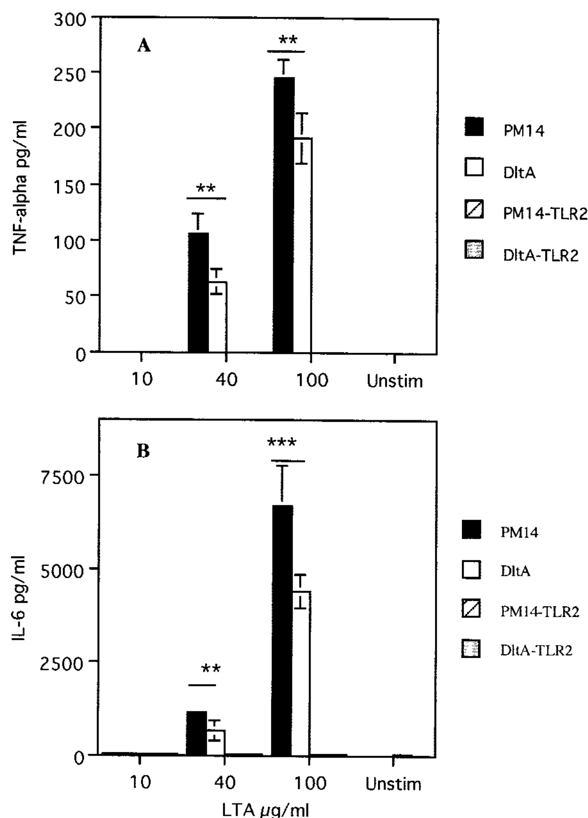

FIG. 5. TNF-α and IL-6 production by 6-day-old bone marrow-derived wild-type dendritic cells in response to stimulation by LTA from *S. gordonii* PM14 or the *dltA* mutant (DltA). TLR2 knockout DCs stimulated PM14 LTA (PM14-TLR2) or *dltA* mutant LTA (DltA-TLR2) produced little or no response. Wild-type and TLR2 knockout DCs stimulated with 1 μg/ml LPS produced 2,500 pg/ml TNF-α and 10,000 pg/ml IL-6 (data not plotted to simplify the graph). Unstim, unstimulated. ***, $P < 0.001$; **, $P < 0.01$.

(Fig. 5). The levels of TNF-α and IL-6 elicited by the *dltA* mutant LTA were significantly lower than that elicited by the wild-type LTA. When TLR2 knockout DCs were stimulated with LTA, no detectable TNF α or IL-6 was observed. Similar to results with the wild-type DCs, IL-12p70 and IL-10 were not produced.

DISCUSSION

Several studies using other gram-positive organisms have demonstrated consistently that D-alanylation mutants are impaired in their ability to resist cationic peptide-mediated killing (9, 15, 19, 32, 33). Not surprisingly, the *S. gordonii dltA* mutant was found to be more susceptible to killing by several cationic peptides, including polymyxin B, nisin, magainin II, and human β defensins 1 and 2 (Fig. 1). Interestingly, the *dltA* mutant did not exhibit increased susceptibility to histatin 5, which is an antimicrobial peptide commonly found in human saliva. It is possible that *S. gordonii* has other mechanisms to protect against lysis by histatin which are not affected by the lack of D-alanylation. Alternatively, it is possible that histatin 5 failed to exert its antimicrobial potential due to an inadequate amount of Zn^{2+} (34) or carbonate (8) present in our assay conditions; however, this remains to be tested.

Despite the previously described inability of an *S. gordonii dltA* mutant to form intragenic coaggregations (4), the mutant used in this study was able to effectively colonize the oral cavity, pharynx, and trachea of BALB/c mice (Table 2). This is in contrast to studies with *S. aureus* that show D-alanine substitution on LTA is essential for colonization in cotton rats (41). The undiminished ability of this bacterium to colonize was not surprising as *S. gordonii* produces a variety of adhesins that mediate binding and colonization such as the antigen I/II family polypeptides SspA and SspB and the sialic acid-binding protein Hsa. These proteins have recently been shown to be important for mediating primary adhesion events by interactions with human cell surface receptors (16).

Initially, it was thought that the D-alanine substitutes on LTA were protecting *S. gordonii* from lysis by host defense peptides naturally found in the oral cavity and that this mechanism prevented the presentation of this bacteria and its expressed antigens from eliciting a strong immune response. However, the present study demonstrates that the lack of D-alanylation on LTA does not affect the mucosal and humoral immune responses to the recombinant *S. gordonii*.

This is the first study of a mutation in the *dlt* operon resulting in altered binding to DCs. Others have previously demonstrated that *dltA* mutants displayed altered adherence to phagocytic cells. A study by Abachin et al. (1) demonstrated that a *dlt* mutant of *Listeria monocytogenes* exhibited decreased adherence to murine bone marrow-derived macrophages. In the current study, the *S. gordonii dltA* mutant displayed a significantly lower level of binding to DCs than its parent, suggesting that D-alanylation plays an important role in modulating the binding of this bacterium to DCs. This change in adherence could be the result of a simple increase in negative charge in the bacterial cell surface causing a decreased interaction with the negatively charged host cells. Our Alcian blue binding assay results showed that the *dltA* mutant has an increased negative charge.

Since it was known from the literature that LTA can directly bind to TLR2 (13, 16, 17), the binding of the *dltA* mutant to TLR2 knockout DCs was examined. The results showed that the parent bound equally well to the normal and the TLR2 knockout DCs. Remarkably, the absence of the TLR2 restored the mutant to wild-type levels of binding. These findings suggest that either the presence of TLR2 inhibits the binding of the *dltA* mutant or, perhaps more plausibly, that the absence of TLR2 allows for better nonspecific or specific interaction of other receptors on the DCs with the bacteria.

Our mouse DCs stimulation studies showed that *S. gordonii* is capable of inducing IL-12p70, TNF-α, IL-6, IL-2, and IL-10 production, which is consistent with a previous study by Corinti et al. (6) demonstrating that human DCs stimulated with a recombinant strain of *S. gordonii* expressing tetanus toxin fragment C induced the release of TNF-α, IL-6, IL-10, and IL-12. Our results further reveal a differential induction of cytokines from DCs by a *dltA* mutant. The mutant exhibited a reduced ability to induce IL-12p70 and IL-10, indicating that D-alanylation plays a role in the response of DCs to *S. gordonii*. However, when purified LTA was used in the stimulation, no detectable IL-12p70 or IL-10 was observed. These results in-

dicate that the observed IL-12p70 and IL-10 induced by *S. gordonii* whole cells was due to other components on the bacteria. The observed reduced ability of the mutant bacteria to induce these two cytokines coincided with the reduced binding to wild-type DCs. In the case of TLR2 knockout DCs, the mutant bacteria induced significantly less cytokines than the parent bacteria, while the binding was similar.

The DC stimulation experiments using purified LTA reveal three interesting findings. First, *S. gordonii* LTA is not capable of inducing IL-12p70 and IL-10. This is in contrast to findings reported by Deininger et al. (7) that purified LTA from *S. aureus* is capable of inducing IL-10 production by human whole blood. Second, *S. gordonii* LTA is capable of inducing TNF-α and IL-6 production, and TLR2 appears to be the receptor. These results are in general agreement with that reported for LTA from other bacteria (7, 14, 15, 35, 36). Third, *S. gordonii* LTA devoid of D-alanine induced significantly less TNF-α and IL-6. This is consistent with findings for purified LTA from group B *Streptococcus* (16) and *S. aureus* (8).

In addition to assessing the induction of cytokines from DCs in response to *S. gordonii* strains, the response to pathogenic group A *Streptococcus* (GAS) was also examined. The responses were compared in an attempt to determine whether there were any particular differences in the cytokine profiles induced, since one organism was able to avoid eliciting an immune response, while the pathogen was certain to elicit a strong one. Previous studies have shown that GAS induces strong Th1-type immune responses in monocytes, macrophages, and streptococci-infected tissues (28, 31, 40). It was shown in this study that GAS induced higher levels of TNF-α than the *S. gordonii* parent strain but not to a level that was statistically different. TNF-α is an inflammatory cytokine, and naturally higher expression of this agent would promote a strong immune response. It is possible that even such small increase in TNF-α production could boost the Th1 response to GAS. GAS also induced significantly higher levels of IL-10 than the *S. gordonii* parent strain but similar levels of IL-6 and IL-12. IL-10 is a tolerance-promoting cytokine, but the expression of other cytokines can overcome its regulatory function; IL-2 is one of those cytokines. It was shown in this study that GAS induces large amounts of IL-2, while *S. gordonii* induces only low levels of IL-2. Low doses of IL-2 have been proven to be essential for the maintenance of regulatory T-cell function (38), but high levels of the same cytokine have been shown to reverse T-cell regulatory suppression and activate the immune response (37, 39). Therefore, the lack of IL-2 induction by *S. gordonii* may contribute to its inability to elicit a strong immune response.

In summary, the results of this study indicate that the D-alanylation of the LTA in *S. gordonii* contributes to relative resistance to cationic peptides, greater DC adherence, and immunomodulation of cytokine production from DCs. Thus, the incorporation of D-alanine into LTA may contribute to the persistence of this organism in the oral cavity by allowing *S. gordonii* to evade host defense peptides, increase contact with DCs that modulate the adaptive response, and subsequently promote a non-Th1 response.

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