Ganglioside GD1a Is an Essential Coreceptor for Toll-like Receptor 2 Signaling in Response to the B subunit of Type IIb Enterotoxin*†

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Innate recognition and signaling by Toll-like receptors (TLRs) is facilitated by functionally associated coreceptors, although the cooperativity mechanisms involved are poorly understood. As a model we investigated TLR2 interactions with the GD1a ganglioside binding subunit of type IIb Escherichia coli enterotoxin (LT-IIb-B₅). Both LT-IIb-B₅ and a GD1a binding-defective mutant (LT-IIb-B₅(T13I)) could modestly bind to TLR2, but only the wild-type molecule displayed a dramatic increase in TLR2 binding activity in the presence of GD1a (although not in the presence of irrelevant gangliosides). Moreover, fluorescence resonance energy transfer experiments indicated that LT-IIb-B₅ induces lipid raft recruitment of TLR2 and TLR1 and their clustering with GD1a, in contrast to the GD1a binding-defective mutant, which moreover fails to activate TLR2 signaling. LT-IIb-B₅-induced cell activation was critically dependent upon the Toll/IL-1 receptor domain-containing adaptor protein, which was induced to colocalize with TLR2 and GD1a, as shown by confocal imaging. Therefore, GD1a provides TLR2 coreceptor function by enabling the ligand to recruit, bind, and activate TLR2. These findings establish a model of TLR2 coreceptor function and, moreover, suggest novel mechanisms of adjuvanticity by non-toxic derivatives of type II enterotoxins dependent upon GD1a/TLR2 cooperative activity.

Recent developments in the field of innate immunity support the concept that cellular activation by microbial molecules involves interactions with multiple cooperating host receptors within membrane lipid rafts, whereas single receptor-based interactions may often represent an oversimplified model (1–3). This concept is exemplified by the ability of pattern-recognition receptors such as Toll-like receptor 4 (TLR4) or TLR2 to functionally associate with accessory molecules or coreceptors for induction of intracellular signaling. TLR4 alone is not sufficient for inducing a vigorous innate response to lipopolysaccharide (LPS) and requires MD-2 and CD14, although additional components of the LPS recognition complex may play a role in modifying TLR4-mediated signaling (4, 5). TLR2 responds to microbial lipoproteins in association with TLR1 or TLR6 as signaling partners and with CD14 or CD36 as important coreceptors for robust activation of TLR2/1 or TLR2/6 complexes (1, 6). The formation of TLR complexes with coreceptors may serve to generate a combinatorial repertoire for discriminating among the abundant and diverse microbial molecules and thereby to appropriately tailor the host response. However, the mechanisms involved in TLR cooperativity with accessory receptors are currently poorly understood.

We have recently shown that the B subunit of type IIb heat-labile enterotoxin of Escherichia coli (designated LT-IIb-B₅) activates TLR2 signaling, although the underlying mechanism was not addressed (7). Type IIb and related enterotoxins (types I and IIa) display an AB₅ oligomeric structure in which a toxic A subunit is noncovalently linked to a pentameric ganglioside binding (B) subunit (8). LT-IIb-B₅ binds avidly to ganglioside GD1a (8, 9). Intriguingly, a GD1a nonbinding mutant (T13I) of LT-IIb-B₅ fails to activate TLR2 (7). These findings imply that GD1a may provide TLR2 coreceptor function for LT-IIb-B₅-mediated cell activation.

In this study we have tested the hypothesis that GD1a and TLR2 cooperate in molecular proximity as a heterotypic receptor complex that is essential for LT-IIb-B₅-induced cellular activation. Mechanistically, GD1a may act by bringing

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The abbreviations used are: TLR, toll-like receptor; LPS, lipopolysaccharide; LT-IIb-B₅, pentameric B subunit of type IIb E. coli enterotoxin; IL, interleukin; ODN, oligodeoxynucleotide; TIRAP, Toll/IL-1 receptor domain-containing adaptor protein; TIRAP-BP, TIRAP-blocking peptide; mAb, monoclonal antibody; CHO, Chinese hamster ovary; HEK, human embryonic kidney; PMP, d,l-threo-1-phenyl-2-hexanecanoylamino-3-morpholino-1-propanol/HCl; MCD, methyl-β-cyclodextrin; FRET, fluorescence resonance energy transfer; YFP, yellow fluorescent protein; CFP, cyan fluorescent protein; FITC, fluorescein isothiocyanate; MHC, major histocompatibility complex; GD1a, Neu5Acα3Galβ3GalNAcβ4(Neu5Acα3)Galβ4Glc- ceramide; GD1b, Galβ3GalNAcβ4(Neu5Acα3)Galβ4Glc-ceramide; GM1, Galβ3GalNAcβ4(Neu5Acα3)Galβ4Glc-ceramide.
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Recombinant wild-type or mutant (T13I) LT-IIb-B₅ were expressed in E. coli DH5α F’ Kan (Invitrogen) transformed with the appropriate plasmids, and the proteins were extracted from the periplasmic space using polymixin B treatment (7, 10). The proteins were purified by means of ammonium sulfate precipitation followed by nickel affinity chromatography and size-exclusion chromatography using a Sephacryl-100 column and an ÄKTA fast protein liquid chromatography system (GE Healthcare). Identity and purity were confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis immunoblotting using specific rabbit IgG antibodies and by quantitative Limulus amebocyte lysate assay kits (BioWhittaker, Walkersville, MD and Charles River Endosafe, Charleston, SC), which determined negligible endotoxic activity (≤0.0064 ng/µg of protein). Further evidence against contamination with LPS or other heat-stable contaminants was obtained upon LT-IIb-B₅ boiling, which destroys their biological activities (Refs. 7 and 10).

EXPERIMENTAL PROCEDURES

Bacterial Molecules and Chemicals

Recombinant wild-type or mutant (T13I) LT-IIb-B₅ were expressed in E. coli DH5α F’ Kan (Invitrogen) transformed with the appropriate plasmids, and the proteins were extracted from the periplasmic space using polymixin B treatment (7, 10). The proteins were purified by means of ammonium sulfate precipitation followed by nickel affinity chromatography and size-exclusion chromatography using a Sephacryl-100 column and an ÄKTA fast protein liquid chromatography system (GE Healthcare). Identity and purity were confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis immunoblotting using specific rabbit IgG antibodies and by quantitative Limulus amebocyte lysate assay kits (BioWhittaker, Walkersville, MD and Charles River Endosafe, Charleston, SC), which determined negligible endotoxic activity (≤0.0064 ng/µg of protein). Further evidence against contamination with LPS or other heat-stable contaminants was obtained upon LT-IIb-B₅ boiling, which destroys their biological activities (Refs. 7 and 10 and current results). Pam3Cys-Ser-Lys4 (Pam3Cys, a synthetic lipopeptide used as a TLR2/TLR1 control agonist) was obtained from EMC Microcollections (Tuebingen, Germany). α,β-threo-1-Phenyl-2-hexadecanoylamino-3-morpholino-1-propanol-HCl (PPMP) and gangliosides (GD1a, GD1b, GM1) and were obtained from Matreya LLC (Pleasant Gap, PA). Methyl-β-cyclohexanecarboxamide (MCHD), cholesterol, cytochalasin D, and brefledomycin A were purchased from Sigma-Aldrich. The reagents were used at effective concentrations determined in preliminary experiments or in previous publications.

Cell Culture

Chinese hamster ovary (CHO)-K1 cells (ATCC CRL-9618) were maintained in Ham’s F-12 nutrient mixture (Invitrogen) supplemented with 2 mM l-glutamine, 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Human embryonic kidney (HEK)-293 cells (ATCC CRL-1573) and COS-7 cells (ATCC CRL-1651) were cultured in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). Monocytes were purified from human peripheral blood upon centrifugation over NycoPrep™1.068 (Axis-Shield, Oslo, Norway) as previously described (11). Incidental non-monocytes were removed by magnetic depletion using a mixture of biotin-conjugated monoclonal antibodies (mAbs) and magnetic microbeads coupled to anti-biotin mAb (Monocyte isolation kit II; Miltenyi Biotech, Auburn, CA). Purified monocytes were cultured at 37 °C and 5% CO₂ atmosphere in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM l-glutamine, 10 mM HEPES, 100 units/ml penicillin G, 100 µg/ml streptomycin, and 0.05 mM 2-mercaptoethanol (complete RPMI). Complete RPMI was also used to culture human monocyteic THP-1 cells (ATCC TIB 202). Thioglycollate-elicited macrophages were isolated from the peritoneal cavity of mice deficient in CD14 or TLR2 or from wild-type C57BL/6 control mice (The Jackson Laboratory, Bar Harbor, Maine) as previously described (7, 12). Mouse macrophages were cultured in complete RPMI, except for the use of autologous mouse serum rather than fetal bovine serum. Cell viability was monitored using the CellTiter-Blue™ assay kit (Promega, Madison, WI). None of the experimental treatments affected cell viability compared with medium-only control treatments. Human blood collections and isolation of mouse macrophages were conducted in compliance with established federal guidelines and institutional policies.

NF-κB Luciferase Assays

Ganglioside-loaded and TLR-transfected CHO-K1 Cells—Reporter assays for NF-κB activation based on inducible luciferase activity were performed as previously described (3, 7). Briefly, CHO-K1 cells were transiently transfected with plasmids encoding human TLR2 (pUNO-HTLR2), a mutant version of TLR2 devoid of the intracellular TIR domain (pZERO-hTLR2), TLR4/MD2 (pDUO-hMD2/TLR4), or empty control vectors (Invivogen; San Diego, CA). To monitor cellular activation, the cells were cotransfected with a firefly luciferase reporter gene controlled by five tandem repeats of NF-κB consensus sequence cloned upstream of a basic promoter (pNF-κB-Luc; Stratagene, La Jolla, CA), along with a Renilla luciferase transfection control (pRL-null; Promega). Transfections were performed using the PolyFect transfection reagent (Qiagen, Inc., Valencia, CA) according to the manufacturer’s instructions. Four hours post-transfection, the cells were incubated with or without GD1a or GD1b (20 µM) in serum-free medium at 37 °C for 24 h. CHO-K1 cells do not synthesize complex gangliosides (13), and the rationale for this treatment was to assess the effect of ganglioside loading on activation of TLR2-transfected CHO-K1 cells by LT-IIb-B₅. At the end of the 24-h incubation, the cells were washed with serum-free medium and stimulated with LT-IIb-B₅ at 37 °C for 6 h. The Renilla and firefly luciferase activities were subsequently measured in cell lysates using the Dual-Glo™ luciferase reporter assay system (Promega) and a Clarity™ luminescence microplate reader.
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(Bio-Tek, Winooski, VT). Luciferase activity was calculated as a ratio of firefly luciferase activity to Renilla luciferase activity to correct for transfection efficiency. The results were then normalized to those of unstimulated control cells transfected with reporter and empty vectors, the value of which was taken as 1.

**HEK-293 and THP-1 Cells**—Similar procedures (except that no ganglioside loading was performed) were followed to determine activation of NF-κB-dependent transcription in ganglioside-expressing HEK-293 cells cotransfected with human TLR1/TLR2 (pDUO-hTLR1/TLR2; InvivoGen) and the NF-κB reporter system described above using FuGENE 6 transfection reagent (Roche Applied Science). NF-κB-dependent transcription in THP-1 cells was assessed as above except that the cells were cotransfected with plasmids expressing dominant negative inhibitors of human TLR2 (pZERO-hTLR2tirless; InvivoGen), TIRAP (pDeNy-hTIRAP; InvivoGen), TIR-domain-containing adapter-inducing IFN-β-related adaptor molecule (TRAM; pDeNy-hTRAM), or empty vector controls.

**TIRAP Inhibition and Cytokine Induction**

Human monocytes or mouse macrophages (2 × 10^5/well) were stimulated with LT-IIb-B₃ for 16 h at 37 °C. Induction of release of IL-6 in culture supernatants was measured by human- or mouse-specific IL-6 enzyme-linked immunosorbent assay kits (eBioscience, San Diego, CA). To determine the role of TIRAP in LT-IIb-B₃-induced cytokine induction, we used a cell-permeable peptide set, including a peptide that inhibits TIRAP binding to human TLR2 or TLR4 and an inactive control peptide (14) (Imgenex; San Diego, CA). For inhibiting mouse TIRAP, we used an analogous cell-permeable TIRAP inhibitory and control peptide set (15, 16) (Calbiochem). The TLR9 agonist, CpG oligodeoxynucleotide (ODN), was used as a TIRAP-independent control. For this purpose, CpG ODN M362 (human-specific) and CpG ODN 1826 (murine-specific) were obtained from InvivoGen.

**Inhibition of Ganglioside Synthesis**

To deplete cellular gangliosides we used the glucosylceramide synthase inhibitor PPMP (17). For this purpose human monocytes were incubated at 37 °C for 3 days with or without 5 μM PPMP. Inhibition of surface expression of ganglioside GD1a was confirmed by reduced reactivity with anti-GD1a mAb or with LT-IIb-B₃ ligand (see “Results”). Untreated and PPMP-treated cells were further incubated (37 °C, 24 h) without or with 20 μM gangliosides (GD1a, GD1b, or GM1) in the absence or presence of 5 μM PPMP. Cells were then washed with culture medium and stimulated (37 °C, 16 h) with the indicated concentrations of LT-IIb-B₃ without or with 5 μM PPMP. Culture supernatants were collected for determining IL-6 induction as described above.

**Binding Assays**

Biotinylated LT-IIb-B₃ (1 μg/ml) was allowed to bind to TLR-transfected and/or ganglioside-loaded CHO-K1 cells or primary human monocytes for 15 min at 37 °C, as we have previously described for other bacterial molecules (12, 18). Subsequently, the cells were washed and incubated on ice with FITC-labeled streptavidin. After washing, binding was determined by measuring cell-associated fluorescence (in relative fluorescence units) on a microplate fluorescence reader (Bio-Tek) with excitation/emission wavelength settings of 485/530 nm. Background fluorescence was determined in cells treated with medium only and FITC-streptavidin. TLR2 binding was measured using a modification of a previously published method (19). Briefly, 96-well microtiter wells were coated overnight at 4 °C with 20 μg/ml purified recombinant mouse TLR2 (R&D systems, Minneapolis, MN). After blocking with 5% (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h at 37 °C, biotinylated wild-type or mutant LT-IIb-B₃ (2 μg/ml in PBS/1% BSA) were allowed to bind to immobilized TLR2 in the absence or presence of gangliosides (10 μM) for 2 h at 37 °C. After washing with phosphate-buffered saline containing 0.05% Tween 20, bound protein was detected with peroxidase-conjugated streptavidin. The peroxidase reaction was performed using tetramethylbenzidine chromogenic substrate, and the optical density signal at 450 nm was read in a microplate reader (Bio-Tek Instruments). Because recombinant TLR2 is expressed as a fusion protein with the Fc region of human IgG (R&D systems), the complete lack of LT-IIb-B₃ binding to recombinant CD14 (supplemental Fig. 1B), which is similarly fused to the Fc region of human IgG, has excluded the possibility of LT-IIb-B₃ binding to the Fc region.

**MCD Treatment and Cholesterol Reconstitution**

To deplete human monocytes of cholesterol using MCD and reconstituting cellular cholesterol in MCD-treated cells we used a modification of previously published methodology (20, 21). Briefly, human monocytes or HEK-293 cells were incubated in the presence of 10 mM MCD for 30 min at 37 °C to deplete the cells of cholesterol. The cells were washed and incubated for an additional 30 min with medium only or with 150 μM cholesterol. Subsequently, the cells (MCD-treated, MCD-treated plus cholesterol-reconstituted, and cells treated with medium only) were incubated with LT-IIb-B₃ for cellular activation assays.

**Fluorescence Resonance Energy Transfer (FRET)**

The procedures for measuring the efficiency of energy transfer between fluorescein-labeled cell surface receptors have been previously described in detail (3, 4, 22). Briefly, human monocytes were cultured on microchamber culture slides (Labtek, Invitrogen). After treatment for 10 min at 37 °C with medium only or with LT-IIb-B₃ (1 μg/ml), the cells were labeled with 100 μl of a mixture of Cy3-conjugated mAb (donor) and Cy5-conjugated mAb (acceptor). The cells were rinsed twice with PBS containing 0.02% bovine serum albumin and then fixed with 4% paraformaldehyde for 15 min. Cell fixation was necessary to prevent potential reorganization of the proteins during the course of the experiment and energy transfer deactivations. Energy transfer between different receptor pairs was calculated from the increase in donor fluorescence after acceptor photobleaching. The following mAbs (clones) were used for FRET: anti-TLR1 (GD2.F4), anti-TLR2 (TL2.1), and anti-TLR4 (HTA-125) were from HyCult (Uden, The Netherlands); anti-GD1a (GD1a-2b) and anti-GD1b (GD1b-1) were from AMS Biotechnology (Oxford, UK); anti-CD14 (Tük 4) and anti-MHC...
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RESULTS

GD1a and TLR2 Cooperate for Cell Activation and Binding of LT-IIb-B$_5$—We investigated the hypothesis that GD1a binding is a necessary condition for efficient TLR2 activation by LT-IIb-B$_5$. For this purpose we used CHO-K1 cells, which do not synthesize GD1a or other complex gangliosides (13). CHO-K1 cells, moreover, lack functional endogenous TLR2 but transcribe TLR1 and TLR6, either of which is capable of cooperative signaling with TLR2 in TLR2-transfected CHO-K1 cells (23, 24). We have, therefore, transfected CHO-K1 cells with human TLR2 to allow reconstitution of TLR2/TLR1 cooperative signaling, required for maximal cell activation by LT-IIb-B$_5$ (7). However, transfection with TLR2 led to relatively poor LT-IIb-B$_5$-induced activation of NF-$\kappa$B-dependent transcription, unless the cells were supplemented with GD1a (Fig. 1A). Indeed, TLR2-transfected and GD1a-supplemented cells exhibited a 4-fold higher NF-$\kappa$B activation in response to LT-IIb-B$_5$ than similarly transfected cells in the absence of GD1a or in the presence of a control ganglioside (GD1b) ($p < 0.05$; Fig. 1A). On the other hand, GD1a-supplemented cells transfected with empty vector or TLR4/MD2 (Fig. 1A) or with a signaling-deficient mutant of TLR2 (devoid of the intracellular TIR domain; TLR2-$\Delta$TIR) (Fig. 1B) failed to activate NF-$\kappa$B in response to LT-IIb-B$_5$. As expected, the control TLR2/TLR1 agonist Pam3Cys activated TLR2-transfected CHO-K1 cells regardless of the presence or absence of gangliosides (Fig. 1A) but did not activate TLR2-$\Delta$TIR-transfected cells (Fig. 1B). The GD1a binding-defective mutant LT-IIb-B$_5$(T13I) (Fig. 1A) as well as boiled LT-IIb-B$_5$ (not shown) failed to activate the cells even when they were supplemented with GD1a and transfected
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with TLR2. These data clearly indicate that effective NF-κB activation by LT-IIb-B5 specifically requires the presence of both GD1α and TLR2.

We next examined the binding of LT-IIb-B5 to CHO-K1 cells upon ganglioside loading and/or TLR transfection. The binding of LT-IIb-B5 was significantly ($p < 0.05$) enhanced upon cell loading with its ganglioside receptor, GD1α, but not with GD1β (Fig. 1C). This finding confirmed the ganglioside specificity of LT-IIb-B5 and that exogenous addition of GD1α is incorporated in the cell membrane. Interestingly, in the absence of GD1α LT-IIb-B5 displayed modest binding to TLR2-transfected cells relative to empty vector-transfected cells (Fig. 1C). The binding of LT-IIb-B5 (used in biotinylated form for probing with streptavidin-FITC) to GD1α-loaded and TLR2-transfected cells was competitively inhibited by excess unlabelled LT-IIb-B5 (not shown). Most importantly, the ability of LT-IIb-B5 to bind plate-immobilized TLR2 was significantly ($p < 0.05$) enhanced in the presence of GD1α but not in the presence of GD1β or GM1 (Fig. 1D). In the absence of GD1α, LT-IIb-B5(T13I) displayed low binding to TLR2, similar to that seen with the wild-type molecule; however, the TLR2 binding activity of LT-IIb-B5(T13I) was not enhanced in the presence of GD1α (Fig. 1D). As expected, control incubation of TLR2 with GD1α alone (i.e., without biotinylated LT-IIb-B5) followed by streptavidin-peroxidase labeling resulted in no signal (Fig. 1D). GD1α (probed with anti-GD1α mAb) displayed negligible binding to TLR2 (not shown). The findings from Fig. 1 collectively indicate that GD1α binding is required for the ability of LT-IIb-B5 to effectively interact with and activate TLR2.

Effect of Native Ganglioside Depletion on LT-IIb-B5-induced Monocyte Activation—To determine the role of native gangliosides on LT-IIb-B5-induced activation of a physiologically more relevant cell type, we treated primary human monocytes with a glucosylceramide synthase inhibitor (PPMP) to deplete cellular gangliosides. We first confirmed that PPMP treatment resulted in reduction of GD1α expression by observing significantly ($p < 0.05$) reduced binding of LT-IIb-B5 or anti-GD1α mAb to treated monocytes compared with untreated controls (Fig. 2A). However, exogenous addition of GD1α, but not of GD1β or GM1, reconstituted the cell binding activity of LT-IIb-B5 or anti-GD1α (Fig. 2A).

In the absence of PPMP treatment, LT-IIb-B5 induced production of IL-6 in a dose-dependent way (Fig. 2B). However, PPMP treatment resulted in significant ($p < 0.05$) inhibition of IL-6 induction by LT-IIb-B5 at all concentrations tested (Fig. 2B). Repletion of GD1α by exogenous addition reversed the inhibitory effect of PPMP on LT-IIb-B5-induced IL-6 release (Fig. 2B), suggesting that the PPMP inhibitory effect is attributed to ganglioside depletion rather than to some nonspecific toxic effect. In contrast to GD1α, GD1β or GM1 repletion failed to reverse the PPMP inhibitory effect (Fig. 2B). These findings confirm the role of GD1α as an essential coreceptor for LT-IIb-B5-induced cellular activation, as seen with transfected cell lines (Fig. 1, A–B).

LT-IIb-B5 Induces Association of TLR2 and TLR1 with Lipid Raft Markers—Lipid rafts can serve as membrane platforms for TLR-mediated signaling (25, 26). Although glycosylphosphatidylinositol-anchored proteins such as CD14 are constitutively found in lipid rafts, TLRs may be recruited there upon activation with appropriate ligands (26, 27). To determine whether TLR2 is recruited to lipid rafts in LT-IIb-B5-stimulated monocytes, we investigated possible TLR2 association with an established lipid raft marker (GM1) using FRET. The ganglioside GM1 is found constitutively and in abundance in lipid rafts, and the use of FRET has demonstrated that GM1 associates with recruitable receptors in correlation with biochemical data on lipid raft composition (26). We observed significant ($p < 0.05$) energy transfer between TLR2 and GM1 in LT-IIb-B5-stimulated cells compared with unstimulated cells, whereas energy transfer between TLR2 and MHC class I (negative control) was consistently low regardless of the cellular activation state (Fig. 3A). These data indicate that LT-IIb-B5 induces TLR2-GM1 association, and therefore, TLR2 is induced to localize within lipid rafts. Moreover, FRET measurements indicated that TLR2 associates with another lipid raft resident molecule, CD14,
upon cell activation (Fig. 3A). However, pretreatment of the cells with MCD, which disrupts lipid raft organization, abrogated energy transfer between TLR2 and GM1 or CD14 (Fig. 3A). Because TLR1 is a TLR2 signaling partner in response to LT-IIb-B₅ (7), we hypothesized that LT-IIb-B₅ would similarly induce association of TLR1 with GM1. Indeed, FRET measurements confirmed this notion, and the specificity of the LT-IIb-B₅-induced TLR1-GM1 association was controlled by the finding that TLR4 failed to associate with GM1 under the same conditions (Fig. 3B). As seen with TLR2 (Fig. 3A), MCD also inhibited the ability of TLR1 to associate with GM1 (Fig. 3B), suggesting that these receptor associations require intact lipid raft function. To rule out the possibility that MCD inhibited energy transfer in a nonspecific way, we measured energy transfer between two different epitopes of the same molecule (CD14) with or without MCD pretreatment. Energy transfer between two distinct CD14 epitopes was consistently high and was not affected by the state of cell activation or the presence of MCD (Fig. 3C; CD14-MHC class I association was used as a negative control for energy transfer). In summary, our findings suggest that cell activation by LT-IIb-B₅ results in mobilization of TLR2 and TLR1 to lipid rafts.

**Lipid Raft Function Is Required for Cell Activation by LT-IIb-B₅**—Membrane lipid rafts are enriched in cholesterol, which plays an important role in maintaining them in a liquid-ordered phase; not surprisingly, depletion of cholesterol by MCD results in disruption of lipid raft organization (20, 26). Because LT-IIb-B₅ induces the association of TLR2 and TLR1 with lipid raft markers, we determined whether cell activation by LT-IIb-B₅ depends upon lipid raft function. For this purpose TLR1/TLR2-transfected HEK-293 cells or primary human monocytes were pretreated at 37 °C for 30 min with 10 mM MCD to deplete the cells of cholesterol. This pretreatment significantly (p < 0.05) inhibited the ability of LT-IIb-B₅ to activate NF-κB-dependent luciferase activity in TLR2/TLR1-transfected HEK-293 cells (Fig. 4A) or to induce IL-6 production in monocytes (Fig. 4B). However, cholesterol repletion of MCD-treated cells completely reversed the inhibitory effects of MCD on NF-κB-dependent transcription (Fig. 4A) or IL-6 induction (Fig. 4B). Therefore, the MCD effect was dependent on cholesterol sequestration and cannot be attributed to nonspecific toxic effects. These findings suggest that
CD14 associates with TLR2 in LT-IIb-B₅-stimulated cells (Fig. 3A) and is moreover a functional coreceptor for TLR2 (1, 3, 22). However, we found that CD14 does not play an accessory role in LT-IIb-B₅-induced and TLR2-mediated cell activation (supplemental Fig. 1A). Indeed, unlike TLR2-deficient mouse macrophages, CD14-deficient macrophages responded to LT-IIb-B₅, similarly to wild-type cells in terms of IL-6 induction (supplemental Fig. 1A). The converse was true for LPS, which was used for control purposes (supplemental Fig. 1A). Moreover, LT-IIb-B₅ failed to bind CD14, in contrast to established CD14 ligands (supplemental Fig. 1B). Therefore, the observed association of CD14 with TLR2 in LT-IIb-B₅-treated cells (Fig. 3A) simply indicates that TLR2 is localized within lipid rafts.

Cell Activation by LT-IIb-B₅ Is Dependent upon TIRAP, Which Co-localizes with Both TLR2 and GD1a—TIRAP (or Mal; MyD88-adapter-like) is an adaptor protein specifically involved in TLR2 (TLR2/1 and TLR2/6) and TLR4 signaling (28, 29). The role of TIRAP is to facilitate the recruitment of MyD88 to the cytoplasmic TIR domain of the activated TLR and thereby activate the MyD88-dependent pathway for NF-κB activation (30). We hypothesized that TIRAP is crucial for mediating LT-IIb-B₅-induced TLR2 signaling, and this was investigated by means of cell activation assays and confocal imaging of LT-IIb-B₅-activated cells. We first determined the ability of human monocytes or mouse macrophages to elicit IL-6 production in response to LT-IIb-B₅, under conditions where the function of TIRAP is blocked. For this purpose we used TIRAP-blocking peptides (TIRAP-BP), which inhibit TIRAP-dependent signaling in human (14) or mouse cells (15, 16). TIRAP-BP, but not the control peptides, significantly (p < 0.05) diminished the ability of LT-IIb-B₅ to induce IL-6 production in human monocytes (Fig. 6A) or mouse macrophages (Fig. 6B). The specificity of the blocking peptides was confirmed by the findings that the peptides did not influence IL-6 induction by a TLR9 agonist, CpG ODN (Fig. 6). These findings were verified in THP-1 cells, the ability of which to respond to LT-IIb-B₅ with NF-κB activation was significantly inhibited by transfection with a dominant negative version of TIRAP (P125H; Ref. 29) or with a signaling-deficient mutant of TLR2 (TLR2-ΔTIR; positive control) (p < 0.05; Fig. 6C). Transfection with a dominant negative version (C117H) of TIR-domain-containing adapter-inducing IFN-B-related adaptor molecule, which is exclusively involved in TLR4 signaling (31), was used as a negative control and showed no effect on LT-IIb-B₅-induced NF-κB-dependent transcription (Fig. 6C).

LT-IIb-B₅ was also used to activate transfected COS-7 cells expressing YFP- and CFP-tagged versions of TLR2 and TIRAP, respectively. In unstimulated cells, CFP-tagged TIRAP exhibited a diffuse distribution in the cytoplasm and did not appear to colocalize with YFP-tagged TLR2, which was expressed on the cell surface as well as intracellularly as indicated by perinuclear concentration of fluorescence (Fig. 7A, left). Upon cell stimulation with LT-IIb-B₅, TLR2 and TIRAP were apparently recruited to and colocalized in the cell membrane (Fig. 7, A, right, and B). Moreover, there was clear colocalization of LT-IIb-B₅ with both TLR2 and TIRAP, as seen in the merged images with extensive overlay areas positive for red (LT-IIb-B₅), green (TLR2), and cyan (TIRAP) fluorescence (Fig. 7, A, right, and B). Because the cell binding activity of LT-IIb-B₅ correlates with that of anti-GD1a mAb (Fig. 2A), LT-IIb-B₅ is a reliable probe for GD1a. Therefore, it can be concluded that GD1a, TLR2, and TIRAP colocalize in LT-IIb-B₅-activated cells and form a receptor/signaling complex mediating cellular activation. Signaling is likely to be initiated at the cell surface, since the internalization of LT-IIb-B₅ and TLR2 was found to be irrelevant to cell activation (supplemental Fig. 2). Confocal microscopy in LT-IIb-B₅-treated COS-7 cells expressing YFP-tagged TLR2 and stained with Cy5-labeled anti-Golgin-97 iden-
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FIGURE 6. LT-IIb-B₅-induced cell activation is TIRAP-dependent. A and B, human monocytes or mouse macrophages were pretreated for 1 h with cell-permeable TIRAP-BP, activated with LT-IIb-B₅ (1 μg/ml) for 16 h, and assayed for induction of IL-6 release. Human-specific TIRAP-BP was used at 40 μM, mouse-specific TIRAP-BP was at 10 μM, and cell-permeable control peptides were used at equal concentrations with corresponding TIRAP-BPs. The specificity of the blocking peptides was controlled using human-specific (A) or mouse-specific (B) CpG ODNs, which activate TIRAP-independent TLR9 signaling. C, human monocyte THP-1 cells were cotransfected with NF-κB-dependent luciferase reporter gene and Renilla transfection control and with plasmids expressing dominant negative (DN) point mutants of human TIRAP (TRAM-DN) or TIR domain-containing adapter-inducing IFN-β-related adaptor molecule (TRAM; TRAM-DN; negative control) or a signaling-deficient mutant of TLR2 devoid of the TIR domain (TLR2-ΔTIR; positive control), at the indicated μg amounts of plasmid DNA per 2 × 10⁵ cells. NF-κB activation is reported as relative luciferase activity (RLA), normalized to that of unstimulated cells transfected with reporter and empty vectors. Data are presented as the means ± S.D. (n = 3) from one of three (A and B) or two (C) independent experiments that yielded similar results. Asterisks indicate statistically significant (p < 0.05) inhibition of cell activation in the indicated assay compared with control treatment.

cated co-localization of the three markers, suggesting that both LT-IIb-B₅ and TLR2 traffic to the Golgi after 30 min of cell stimulation (supplemental Fig. 2A). However, cell pretreatment with cytochalasin D (which inhibits internalization) or with brefeldin A (which inhibits targeting to the Golgi) had no sig-
ficant effects on the ability of LT-IIb-B₅ to induce NF-κB activation (supplemental Fig. 2B) or IL-6 production (supple-
mental Fig. 2C).

DISCUSSION

Using a variety of independent approaches (biochemical, immunological, biophysical, and cell imaging), we generated evidence establishing ganglioside GD1α as a novel coreceptor of TLR2. Indeed, the presence of GD1α was instrumental for maximal TLR2 binding and TLR2-dependent NF-κB activation by LT-IIb-B₅. Other TLR2 coreceptors include CD14 (32), CD36 (33), the mannose receptor (34), and dectin-1 (35). These and other as yet unidentified innate immune receptors may assist TLR2 and its signaling partners (TLR1 or TLR6) to recognize and respond to a plethora of microbial molecules. The identification of an increasingly diverse array of TLR2 coreceptors appears to account, at least partially, for the remarkable promiscuity of TLR2 in ligand recognition. Indeed, compared with other TLRs, TLR2 appears to have the largest repertoire of microbial ligands, including peptidoglycan, lipoteichoic acid, lipoarabinomannan, lipoproteins, glycolipids, and zymosan as well as atypical LPS from certain Gram-negative bacteria (for review, see Ref. 36).

Our results are summarized in Fig. 8, which presents a working model for LT-IIb-B₅-induced GD1α/TLR2/1 cooperation. The LT-IIb-B₅ ligand and its receptors along with TIRAP colocalize and initiate downstream signaling for NF-κB activation. The finding that the TLR2 binding activity of LT-IIb-B₅ is greatly enhanced in the presence of GD1α (Fig. 1D) suggests that GD1α/LT-IIb-B₅ complexes bind more avidly to TLR2 than LT-IIb-B₅, or GD1α alone. In this regard, the propensity of TLR2 and its sig-

naling partners to respond to cer-
tain lipids attached to proteins or
peptides (37, 38) or to glycolipids (39) may enable TLR2 to recognize
GD1α-bound LT-IIb-B₅, as a glyco-
lipid-protein complex. Alterna-
tively, the binding of LT-IIb-B₅ to
GD1α may induce a conformational change in LT-IIb-B₅ that enables it
to bind more avidly to TLR2. Engagement of GD1α/LT-IIb-B₅ by TLR2 may target this TLR to lipid rafts, which are enriched in ganglio-
sides including GD1α (40, 41). We believe that at least one mechanism whereby a lipid can recruit and retain a TLR to lipid rafts involves the ability of the ligand to engage both a lipid raft molecule (GD1α, in the case of LT-IIb-B₅) and the TLR in question. Unlike LT-IIb-B₅, which binds both GD1α and TLR2, LT-IIb-B₅ (T13I) does not bind GD1α and, thus, cannot induce formation of GD1α/TLR2 complexes in lipid rafts or for that matter initiate activating signals.

The specific mechanism whereby LT-IIb-B₅ may interact with TLR2 is uncertain, although some insights may be gleaned from its crystallographic structure. LT-IIb-B₅ and other B pen-
tamers of heat-labile enterotoxins can participate in both hydrophilic and hydrophobic interactions (42, 43). The so-called “lower end” of the B pentamer pore of LT-IIb interacts through hydrophilic interactions with the extracytoplasmic oligogalactoside moiety of GD1α, which is anchored to the cell membrane via an intramembrane ceramide lipid. The “upper end” of the B pentamer pore contains a large hydrophobic surface (549 Å²) that engages in hydrophobic interactions with the A2 subcomponent of the A subunit; however, this hydrophobic surface area is solvent-accessible in the absence of the A subunit (42, 43). Because of the absence of an A subunit, LT-IIB₅ might, therefore, be free to interact with TLR2 or other recep-
tors that depend on hydrophobic interactions for ligand binding (44–46). If this model is correct, LT-IIb-B₅ may interact
concomitantly with GD1a (via its lower end region) and with TLR2 (via its upper end region). This possibility is consistent with our findings, since GD1a not only does not compete with TLR2 but assists TLR2 for binding LT-IIb-B5 (Fig. 1D).

In addition to our findings, independent evidence from recent reports further supports the general concept that gangliosides interact with TLRs. Indeed, asialoGM1 was shown to cooperate with TLR2 or TLR5 for induction of IL-8 in human corneal or lung epithelial cells (25, 47). In these experimental models it is thought that bacterial ligands are captured by high affinity interactions with membrane-anchored gangliosides, but signal transduction requires physically associated TLRs. Gangliosides recognize diverse microbial structures and may, thus, possess pattern recognition capabilities. For example, in addition to the LT-IIb toxin, GD1a recognizes the *Pseudomonas aeruginosa* flagellin (48) and the *Polyomavirus* (49). Similarly, other enterotoxin signaling receptors for induction of cellular responses.

Until recently it was thought that the immunomodulatory and adjuvant effects of type II or other heat-labile enterotoxins (such as type I toxins from *E. coli* or *Vibrio cholerae*) depend upon their ganglioside binding and catalytic-toxic activities, the latter precluding the use of intact holotoxins as adjuvants for human use (for review, see Ref. 56). However, the catalytic activity of the A subunit may not be critical for adjuvant action, and in this regard the isolated non-toxic B pentamers do display immunomodulatory activity (for reviewed, see Ref. 56). The ganglioside binding activity of the B pentamers is important but not sufficient for their immunomodulatory effects, since certain point mutations that do not preclude ganglioside binding result in defective immunomodulatory action (57, 58). These findings suggest that additional receptors may be required for optimal immunomodulatory signaling by enterotoxin B pentamers. In the
case of LT-IIb-B5, such previously unidentified signaling receptor may be the TLR2, which may add an additional level of complexity regarding modes of adjuvant action by the enterotoxin family of molecules. LT-IIb-B5 may conceivably promote adaptive immune responses through GD1a/TLR2-dependent induction of costimulatory molecules and immuno-enhancing cytokines by antigen-presenting cells (59), a possibility that is currently under investigation.

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FIGURE 8. Model for GD1a-TLR cooperation in cellular activation by LT-IIb-B5. GD1a binds LT-IIb-B5 and facilitates its interaction with the TLR2/TLR1 signaling complex, which is recruited to lipid rafts. Induction of TLR2/TLR1 signaling for NF-κB activation requires the adaptor protein TIRAP, which colocalizes with the LT-IIb-B5 receptor complex (GD1a/TLR2/TLR1).
