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In vivo and in vitro adjuvant activities of the B subunit of Type IIb heat-labile enterotoxin (LT-IIb-B₅) from *Escherichia coli*

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Abstract

The pentameric B subunit of the *Escherichia coli* LT-IIb enterotoxin (LT-IIb-B₅) activates TLR2 signaling in macrophages. Herein we demonstrate that LT-IIb-B₅, in contrast to a TLR2-nonbinding point mutant, induces functional activation of bone marrow-derived dendritic cells and stimulates CD4⁺ T cell proliferation, activities which suggested that LT-IIb-B₅ might function as an adjuvant in vivo. Indeed, in an intranasal mouse immunization model, LT-IIb-B₅ augmented specific mucosal and serum antibody responses to a co-administered immunogen, at levels which were almost comparable to those induced by intact LT-IIb holotoxin, a potent but toxic adjuvant. Therefore, LT-IIb-B₅ displays useful adjuvant properties which, combined with lack of enterotoxicity and relative stability against degradation, may find application in mucosal vaccines.

Keywords

Heat-labile enterotoxins; mucosal adjuvants; dendritic cells

1. Introduction

The heat-labile enterotoxins of *Escherichia coli* and *Vibrio cholerae* are structurally related and can be classified into two major types based on genetic, biochemical, and immunological properties [1]. The Type I subfamily includes cholera toxin and *E. coli* LT-I, whereas the Type II subfamily comprises the antigenically cross-reactive *E. coli* LT-IIa and LT-IIb [1,2]. Both Type I and Type II enterotoxins display a similar AB₅ oligomeric structure, wherein an enzymatically active and toxic A subunit is noncovalently inserted into the pore of the doughnut-shaped B pentameric subunit [3,4]. The B pentamer in itself is nontoxic but mediates intracellular delivery of the A subunit following high-affinity binding to membrane gangliosides. The internalized A subunit subsequently catalyzes ADP-ribosylation of the

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Gs α component of adenylate cyclase, leading to dramatic and unregulated elevation of intracellular cAMP [1]. In intoxicated gut epithelial cells, cAMP elevation results in massive secretion of electrolytes and water into the gut lumen, clinically manifested as diarrhea [1].

The heat-labile enterotoxins have attracted considerable attention due to their exceptional mucosal adjuvant properties [5], although their intrinsic enterotoxicity precludes their use as adjuvants for human vaccines. It therefore became imperative to identify immunoenhancing activities that can be separated from the enzymatic/toxic activity of the A subunit, and this has been the subject of intensive investigation [6–8]. Our own efforts have focused on the LT-II toxins, which possess immunostimulatory properties that are quite distinct from those of cholera toxin and LT-I (reviewed in refs. [2,7]).

In a study examining innate immune interactions of LT-II toxins and their B pentamers, we found that the latter activate nuclear factor (NF)- κ B, whereas the intact molecules do not [9]. In subsequent studies, the ability of the LT-II B pentamers to activate NF- κ B (and induce production of NF- κ B-dependent cytokines) was attributed to stimulation of Toll-like receptor 2 (TLR2) [10,11]. Intriguingly, the NF- κ B inducing activity of the B pentamer of LT-IIb (designated LT-IIb-B₅) is strongly antagonized by the LT-IIb holotoxin, although not by catalytically defective point mutants [12]. This implied that the antagonistic mechanism is cAMP-dependent, which was confirmed in control experiments using a permeable cAMP analog or a cAMP synthesis inhibitor [12]. It is thus conceivable that the demonstrated mucosal adjuvanticity of the LT-IIb holotoxin [13] may be exerted under relatively non-inflammatory conditions, as previously suggested for cholera toxin [14,15]. Also implicit in the findings on NF- κ B activation by LT-IIb-B₅ [10,12] was the notion that this B pentamer may display NF- κ B-dependent adjuvant activities, such as induction of costimulatory molecules and immunoenhancing cytokines in antigen-presenting cells [16,17].

In this study, we examined whether LT-IIb-B₅ can induce maturation and activation of bone marrow-derived dendritic cells (BM-DC) in a way that could provide functional costimulation to CD4⁺ T cells. Moreover, using an established mouse mucosal immunization model, we investigated whether LT-IIb-B₅ can promote specific antibody responses to a co-administered protein immunogen, namely the AgI/II adhesin from *Streptococcus mutans* [18,19]. Our findings indicate that LT-IIb-B₅ displays useful adjuvant properties which, combined with lack of enterotoxicity and relative stability against degradation [1,2,7], suggest its potential for use in mucosal vaccines.

2. Materials and Methods

2.1. Enterotoxins and other reagents

The construction of recombinant plasmids encoding His-tagged versions of wild-type LT-IIb or LT-IIb-B₅ has been previously described [9]. A single-point substitution mutation (S74D) in the LT-IIb-B₅ was engineered by means of site-directed mutagenesis (QuikChange® kit, Stratagene, La Jolla, CA). LT-IIb-B₅ and derivatives were expressed in *E. coli* DH5 α F' Kan (Life Technologies, Gaithersburg, MD) transformed with the appropriate plasmids, and the proteins were extracted from the periplasmic space using polymyxin B treatment [9,10]. 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-FPLC system (Pharmacia, Piscataway, NJ). The AgI/II protein adhesin was purified from culture supernatants of *S. mutans* by means of size exclusion and anion exchange chromatography, as previously described [19]. Identity and purity of the proteins were confirmed by SDS-PAGE, immunoblotting using specific rabbit IgG antibodies, and by quantitative *Limulus* amebocyte lysate assay kits (BioWhittaker, Walkersville, MD or Charles River Endosafe, Charleston, SC) which determined negligible endotoxic activity (< 0.007 ng/

µg protein). The Pam3Cys-Ser-Lys4 lipopeptide (Pam3Cys) and *E. coli* LPS (ultrapure grade) were purchased from InVivogen (San Diego, CA). The reagents were used at effective concentrations determined in preliminary experiments or in previous publications [9–12,20].

2.2. Cell isolation and culture

Bone marrow-derived dendritic cells (BMDC) were generated as described by Lutz et al [21]. Briefly, bone marrow cells from femurs and tibia of 8–12-week-old mice were plated at 2×10^5 cells/ml and cultured at 37°C and 5% CO₂ atmosphere, in complete RPMI (RPMI 1640 containing 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; InVitrogen, Carlsbad, CA) supplemented with 20 ng/ml recombinant murine GM-CSF (Peprotech, Rocky Hill, NJ). The nonadherent cells were harvested on day 8 and were phenotypically characterized by flow cytometry (see below). The generated BMDC were cultured in complete RPMI. Cell viability was monitored using the CellTiter-Blue™ assay kit (Promega, Madison, WI). None of the experimental treatments affected cell viability compared to medium-only control treatments. The mice used for generating BMDC included wild-type BALB/c or C57BL/6 (The Jackson Laboratory, Bar Harbor, ME) or C57BL/6 MyD88^{-/-} mice (kindly provided by Dr. Shizuo Akira, Osaka University, Japan). All animal procedures associated with tissue harvesting were approved by the institutional animal care and use committee, in compliance with established federal and state policies.

2.3 Quantitative real-time PCR arrays

Gene expression was quantified using a TLR signaling pathway-focused array following the manufacturer's protocol (RT² profiler arrays; SuperArray Bioscience, Frederick, MD). Briefly, BMDC were stimulated with LT-IIb-B₅ and RNA was extracted from cell lysates using the PerfectPure RNA cell kit (5 Prime, Fisherscience, Pittsburg, PA). The RNA was reverse-transcribed and quantitative real-time PCR with cDNA was performed using an ABI 7500 system (Applied Biosystem, Foster City, CA). BMDC without stimulation were used as control. The data were analyzed using a Web-based PCR Array Analysis software by SuperArray Bioscience.

2.4. Cytokine induction

BMDC were stimulated with LT-IIb-B₅ or control agonists and collected supernatants were analyzed for cytokine induction using ELISA kits (eBioscience) [22].

2.5. FACS, antibodies, and costimulatory molecule expression in BMDC

The phenotypic characterization of the generated BMDC and determination of costimulatory molecule upregulation in activated BMDC were performed by flow cytometric analysis, using the FACSCalibur and the CellQuest software (Becton–Dickinson) [23]. For these experiments, we used fluorescently labeled monoclonal antibodies to the following cell surface markers: CD11c (clone HL3); CD11b (M1/70); CD40 (HM40-3); CD54 (YN1/1.7.4); CD80 (16-10A1); CD86 (GL1); I-A/I-E (M5/114.15.2); Gr1 (RB6-8C5); and F4/80 (BM8). All antibodies and isotype controls were from eBioscience except for anti-CD11c from BD Pharmingen. The cells were pretreated with Fc Block CD16/32 (clone 93) and subsequently labeled with antibodies in “staining” buffer (Dulbecco's PBS containing 0.1% BSA and 0.01% azide), followed by washing and flow cytometry. The phenotypic analysis of the harvested BMDC revealed the presence of a highly pure CD11c⁺ population with <1% macrophages and <5% granulocytes.

2.6. T cell proliferation

CD4⁺ helper T cells were purified from splenocytes of 6–8 week-old BALB/c or C57BL/6 mice using the autoMACS™ separator and anti-CD4⁺ beads (Miltenyi Biotec, Auburn, CA).

T cell proliferation was assessed by flow cytometry (BD FACSCalibur) after a two-day co-culture of carboxyfluorescein succinimidyl ester (CFSE)-stained T cells with LT-IIb-B₅-stimulated or unstimulated BMDC, in the presence of suboptimal concentration (30ng/ml) of anti-CD3 (145-2C11; BD Biosciences). To accurately evaluate the effects of LT-IIb-B₅ on BMDC-mediated proliferation of helper T cells, the BMDC used in the co-culture system were previously irradiated to prevent their own proliferation and were extensively washed to remove residual LT-IIb-B₅. In some experiments, T cell proliferation was determined using the BrdU cell proliferation assay kit, as recommended by the manufacturer (Calbiochem, San Diego, CA). Briefly, stimulated or unstimulated cells were irradiated and co-cultured with CD4⁺ helper T cells and BrdU label. Twenty-four hours later, BrdU incorporation was assessed by intracellular staining with anti-BrdU antibody, followed by peroxidase-conjugated secondary antibody. The peroxidase reaction was performed using tetramethyl benzidine chromogenic substrate and the optical density signal at 450 nm was read on a microplate reader (Bio-Tek Instruments, Winooski, VT).

2.7. Mouse model of mucosal immunization

The use of animals was reviewed and approved by the Institutional Animal Care and Use Committee, in compliance with established Federal and State guidelines. Groups of 6 female BALB/c mice, 10 to 12 weeks old, were used for intranasal immunization, essentially as previously described [20,24]. AgI/II, a protein adhesin from *Streptococcus mutans* [18], was used as the immunogen, either alone or admixed with LT-IIb-B₅ or LT-IIb holotoxin (positive control adjuvant). The mice were administered three doses of AgI/II (10 µg) with or without LT-IIb-B₅ or LT-IIb (both at 1 µg), or buffer only (sham immunized) at 14-day intervals, *i.e.*, at days 1, 15, and 29. The immunogen/adjuvant mixture was slowly administered in a standardized volume (15 µl) to the external nares by means of a micropipettor. Serum was obtained by centrifugation of blood samples collected from the tail vein. Saliva samples were collected by means of a pipettor fitted with a plastic tip after stimulation of the salivary flow by intraperitoneal injection of 5 µg carbachol. Vaginal wash samples were collected by instilling 75 µl of sterile PBS with a pipettor and tip, and flushing three times. Preimmune samples were obtained one day before the immunizations (and confirmed the lack of AgI/II-specific antibodies) and post-immunization collections were made one week after the second and third immunization, as well as two weeks after the third immunization (*i.e.*, at days 22, 36, and 50). Serum and secretions were stored at -80°C until assayed. The levels of isotype-specific anti-AgI/II antibodies from serum and secretions were determined by ELISA on microtiter plates coated with 1 µg/ml AgI/II. Total S-IgA was determined on plates coated with goat anti-mouse IgA. The plates were developed with the appropriate peroxidase-conjugated goat anti-mouse Ig isotype (Southern Biotechnology Associates, Inc., Birmingham, AL) and tetramethyl benzidine chromogenic substrate. Optical density values were measured in an ELISA plate reader. The assay was calibrated by means of a serially diluted standard (Mouse Ig Reference Serum; ICN, Costa Mesa, CA) and a standard curve was generated by a computer program based on four-parameter logistic algorithms. Antibody data were expressed in µg/ml (serum) or % specific antibody/total IgA (secretions).

2.8 Statistical analysis

Data were evaluated by analysis of variance and the Dunnett multiple-comparison test using the InStat v3.06 program (GraphPad Software, San Diego, CA). $P < 0.05$ was taken as the level of significance.

3. Results

3.1. LT-IIb-B₅- induced gene expression profile in BMDC

TLR-mediated activation of dendritic cells represents an important mechanism of adjuvant action [16,25]. Since LT-IIb-B₅ can bind and activate the TLR2/TLR1 heterodimer [10,11], we performed a TLR pathway-focused real-time PCR array in LT-IIb-B₅-stimulated BMDC to get an insight on how LT-IIb-B₅ may modulate these cells for adjuvant function. For gene expression analysis, stimulated BMDC from BALB/c mice were compared with control unstimulated cells (Table 1). The threshold line for significant upregulation was set to 3-fold higher above unstimulated expression. Among the significantly upregulated genes, most were found to be genes for cytokines (Il12a, Il6, Tnf, Il1b, Il1a, and Il10), which were upregulated at least five-fold (Table 1). Similar upregulations (though somewhat different quantitatively) were observed in LT-IIb-B₅-stimulated BMDC from C57BL/6 mice (Table 1). However, gene expression upregulation was abrogated in stimulated MyD88-deficient BMDC (Table 1), consistent with the requirement of MyD88-dependent signaling for TLR2 agonists such as LT-IIb-B₅. The growth factor G-CSF (Csf3), cyclooxygenase-2 (Ptgs2), and the macrophage-inducible C-type lectin (Clec4e) were also highly induced by LT-IIb-B₅ (Table 1). Other increases in expression included genes for Toll-like receptors (Tlr1, Tlr2) or co-receptors (Cd14), as well as CD80, an important co-stimulatory molecule (Table 1). The ability of LT-IIb-B₅ to induce a number of immunostimulatory genes implies that it may be capable of enhancing the antigen-presenting function of dendritic cells. This possibility, *i.e.*, that LT-IIb-B₅ may induce cytokines and co-stimulatory molecules at the protein level and enable BMDC to activate T cells, was addressed in the following experiments.

3.2. LT-IIb-B₅ induces maturation and activation of BMDC

We first examined the capacity of LT-IIb-B₅ to stimulate cytokine production in BMDC. Stimulation of BMDC with LT-IIb-B₅ resulted in production of TNF- α and IL-6 in both agonist dose-dependent and cell dose-dependent manner (Fig. 1A-B and C-D, respectively). In contrast to LT-IIb-B₅ or prototypical TLR2 (Pam3Cys) or TLR4 (LPS) agonists, a TLR2-nonbinding point mutant (S74D) of LT-IIb-B₅ [26] failed to induce cytokine production (Fig. 1, C–D). Consistent with its demonstrated inability to induce cytokine gene expression in MyD88^{−/−} BMDC (Table 1), LT-IIb-B₅ failed to stimulate TNF- α or IL-6 production in MyD88^{−/−} BMDC (Fig. 2). Although MyD88 is critical for cytokine induction by TLR2 agonists, TLR4 agonists may additionally stimulate cytokine production in a MyD88-independent way [27]. Indeed, in control experiments with MyD88^{−/−} BMDC, LPS (but not Pam3Cys) retained partial cytokine-inducing activities (Fig. 2); this confirmed that the lack of LT-IIb-B₅-induced responsiveness of MyD88^{−/−} BMDC was not due to any intrinsic defects of these cells in our culture system.

We next determined the ability of LT-IIb-B₅ to upregulate expression of class II MHC and costimulatory molecules. For this purpose, BMDC were harvested as early as 7 days after incubation with GM-CSF in order to obtain immature dendritic cells and prevent further maturation [21]. We found that LT-IIb-B₅ (as well as LPS; positive control) enhanced the expression of class II MHC as well as of CD80 and CD86 (Fig. 3), which are necessary for induction of T cell proliferation through CD28 signaling [28]. CD40, which is important for DC maturation and induction of adaptive immunity [29], was also upregulated and so was CD54 (Fig. 3), which contributes to optimal T cell activation through LFA-1 interaction [30]. Moreover, LT-IIb-B₅ caused moderate upregulation of inducible T-cell costimulator-ligand (ICOSL; CD275) (Fig. 3), which is known to contribute to Th2 cell development by interacting with ICOS, a CD28-related molecule on T cells [31].

3.3. LT-IIb-B₅-stimulated BMDC promote helper T cell proliferation

We next determined whether upregulation of costimulatory molecule expression on BMDC by LT-IIb-B₅ results in functional costimulation of co-cultured CD4⁺ T cells. In these experiments, BMDC were treated or not with wild-type LT-IIb-B₅ or the S74D mutant (negative control), prior to coculture with CFSE-stained CD4⁺ T cells in the presence of suboptimal concentration of anti-CD3. FACS analysis of CD4⁺ T cell division demonstrated that LT-IIb-B₅-treated BMDC promoted T cell proliferation, in contrast to the S74D mutant the effect of which was indistinguishable from the medium-only control (Fig. 4A). The ability of LT-IIb-B₅ to activate BMDC for inducing CD4⁺ T cell proliferation was also tested using an independent method (BrdU incorporation) (Fig. 4B). Again, in contrast to the S74D mutant, wild-type LT-IIb-B₅ (as well as Pam3Cys or LPS; positive controls) promoted BMDC-induced proliferation of CD4⁺ T cells (Fig. 4B).

3.4. Mucosal adjuvanticity of LT-IIb-B₅

The above documented in vitro immunostimulatory activities LT-IIb-B₅ suggest its potential to function as a vaccine adjuvant. To determine whether LT-IIb-B₅ indeed displays mucosal adjuvant capacity, as previously shown for the LT-IIb holotoxin [13], groups of mice were immunized intranasally with *S. mutans* protein AgI/II (10 µg), in the absence or presence of LT-IIb-B₅ or LT-IIb (both at 1 µg), as outlined in the *Methods*. In general, mice given AgI/II with LT-IIb-B₅ or LT-IIb elicited significantly ($p < 0.05$) higher mucosal and systemic AgI/II-specific antibody responses than mice immunized with AgI/II alone (Fig. 5). Interestingly, the capacity of LT-IIb-B₅ to stimulate the salivary IgA response to AgI/II was comparable to that of LT-IIb (Fig. 5A; days 36 and 50), although the holotoxin was the most potent adjuvant in augmenting vaginal IgA (Fig. 5B) or serum IgG (Fig. 5D) responses. Moreover, LT-IIb-B₅ and LT-IIb displayed similar abilities in promoting the serum IgA antibody responses at the earlier time points examined (Fig. 5C; days 22 and 36). These data clearly demonstrate that LT-IIb-B₅ can potentiate specific antibody responses to a mucosally co-administered protein antigen.

4. Discussion

Induced production of immunostimulatory cytokines and upregulation of costimulatory molecules in dendritic cells are required for effective activation of T cells and prevention of tolerance [32,33]. Although TLR2 can function as an adjuvant receptor [34,35], engagement of dendritic cell TLR2 by microbial ligands does not necessarily result in immunostimulation. Indeed, TLR2 signaling may also lead to the generation of regulatory dendritic cells and induction of T cell tolerance, which probably depends on the nature of the TLR2 ligands and their propensity to concomitantly engage other receptors that may crosstalk with TLRs [36]. Nevertheless, LT-IIb-B₅ does activate dendritic cells, and this is manifested as upregulated expression of CD40, CD54, CD80, CD86, MHC Class II, production of TNF- α and IL-6, and proliferation of co-cultured CD4⁺ T cells. Moreover, LT-IIb-B₅ upregulates surface expression of maturation/costimulatory markers (CD83, CD80, CD86) in human monocyte-derived dendritic cells (our unpublished observations). These immunomodulatory mechanisms may, at least in part, be responsible for the observed ability of LT-IIb-B₅ to function as an adjuvant in vivo.

Although LT-IIb-B₅ binds the GD1a ganglioside, which enhances its ability to induce TLR2/1-mediated NF- κ B activation [11], GD1a does not seem to regulate signal transduction. The role of GD1a is to facilitate the LT-IIb-B₅ interaction with TLR2/TLR1 heterodimer, probably by concentrating the agonist in lipid rafts and rendering it more available to the TLR2/TLR1 signaling complex [11]. Consistent with these notions, the TLR2-nonbinding S74D point mutant failed to activate BMDC despite maintaining full GD1a-binding capacity. The lack of

immunostimulatory activity by S74D also confirms that TLR2 signaling is required for BMDC activation by LT-IIb-B₅, which for that matter fails to stimulate cytokine production in cells lacking MyD88, an essential TLR2 signaling adaptor [37]. Interestingly, significant upregulation of TLR2 and TLR1 mRNA expression was observed in LT-IIb-B₅-stimulated BMDC, which could further contribute to the activation of these cells by LT-IIb-B₅.

Although LT-IIb antagonizes TLR2-induced NF- κ B activation by LT-IIb-B₅ in a cAMP-dependent mode [12], induction of proinflammatory signaling is not an obligatory mechanism for adjuvanticity [7,38,39]. Cholera toxin, a potent cAMP-inducing enterotoxin and mucosal adjuvant [5], is noninflammatory at doses that readily induce intestinal fluid secretion in animals [14], whereas, in humans, the expression or not of cholera toxin by *V. cholerae* strains determines whether they induce, respectively, noninflammatory diarrhea or inflammatory gastroenteritis [15]. In terms of immunological properties, cholera toxin promotes the induction of Type 1 regulatory T cells as well as Th2 cells, whereas it proactively inhibits Th1 cell differentiation and induction of proinflammatory cytokines, such as IL-12 and TNF- α [7,38,40,41]. Moreover, it enhances antigen presentation and promotes immunoglobulin isotype differentiation in B cells, leading to enhanced mucosal IgA production [38,40–42]. In an analogous manner, the LT-IIb holotoxin may exert adjuvant action in a non-inflammatory manner mediated by its ganglioside-binding and toxic-catalytic activities, although Type I and Type II holotoxins arguably contain A subunit-dependent adjuvanticity that is independent of catalytic activity (reviewed in refs. [2,7,43,44]). In contrast, LT-IIb-B₅ may conceivably enhance adaptive immunity through TLR2-induced NF- κ B-dependent activities and, therefore, the adjuvant activities of LT-IIb and LT-IIb-B₅ appear to be exerted through distinct mechanisms.

Having established an in vitro mechanistic basis for the adjuvant action of LT-IIb-B₅ and having determined that LT-IIb-B₅ acts as a mucosal adjuvant in vivo, our future studies will focus on establishing the in vivo adjuvant mechanisms of LT-IIb-B₅ and its potential for inducing protective responses against infection. Although LT-IIb was found in this study to be a generally more potent adjuvant than LT-IIb-B₅ (especially for inducing serum IgG antibodies), the lack of enterotoxic side-effects by LT-IIb-B₅ is an overriding advantage for future clinical applications. Strikingly, however, the ability of LT-IIb-B₅ to stimulate salivary IgA antibody responses to *S. mutans* AgI/II was found to be comparable to that of LT-IIb. This finding is important given that *S. mutans* is a major oral pathogen involved in the pathogenesis of dental caries and its AgI/II adhesin constitutes a protective antigen target [45]. Thus, LT-IIb-B₅ can be considered as a novel adjuvant for caries vaccination, although its ability to stimulate IgA responses at remote mucosal sites (*e.g.*, vaginal IgA response) suggests its potential for possible use in vaccine formulations against pathogens which invade via a variety of mucosal routes.

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Fig. 1. Agonist dose- and cell dose-dependent cytokine induction by LT-IIb-B₅ in BMDC
 BMDC were stimulated with LT-IIb-B₅ (A–D) or a TLR2-nonbinding mutant (S74D) (C–D). Pam3Cys (100ng/ml) and LPS (100ng/ml) were used as positive controls (C–D). Induction of TNF-α (A,C) or IL-6 (B,D) production in culture supernatants was measured by ELISA. A and B show agonist dose-dependent cytokine induction, whereas C and D show BMDC dose-dependent cytokine induction. Asterisks indicate significantly ($p < 0.05$) higher cytokine production by stimulation compared to no agonist stimulation. Data are means with SD ($n = 3$) from one of three independent sets of experiments yielding similar results. Asterisks indicate significantly ($p < 0.05$) enhanced cytokine production compared to medium-only stimulation.

Fig. 2. MyD88-dependent cytokine induction by LT-IIb-B₅ in BMDC

BMDC from wild-type or MyD88^{-/-} mice were stimulated with LT-IIb-B₅ or a TLR2-nonbinding mutant (S74D) (10μg/ml). Pam3Cys (100ng/ml) and LPS (100ng/ml) were used as positive controls. Induction of TNF-α (A) or IL-6 (B) production in culture supernatants was measured by ELISA. Data are means with SD (*n* = 3) from one of two independent experiments yielding similar results. Asterisks indicate significantly (*p* < 0.05) enhanced cytokine production compared to medium only.

Fig. 3. Upregulation of class II MHC and of costimulatory molecule expression in BMDC by LT-IIb-B₅

BMDC were incubated with medium only or stimulated for 16 h with LT-IIb-B₅ (10 µg/ml) and expression of the indicated surface molecules was assayed by flow cytometry. BMDC stimulated with LPS (100 ng/ml) were used as positive controls. The histograms shown are from one of five independent sets of experiments that yielded similar findings. Numbers in the histograms are mean fluorescent intensity values.

Fig. 4. Induction of CD4⁺ T cell proliferation by LT-IIb-B₅-stimulated BMDC

Mouse BMDC were stimulated or not for 16 h with wild-type (WT) or mutant (S74D) LT-IIb-B₅ (10 µg/ml). After washing three times with culture medium, the stimulated BMDC were co-cultured with CFSE-stained CD4⁺ T cells in the presence of suboptimal concentration of anti-CD3. After staining with PE-Cy5-anti-CD4, proliferation of gated CD4⁺ T cells was analyzed by FACS (A). In a similar co-culture experiment, CD4⁺ T cell proliferation was assessed using the BrdU assay, where the results are presented as % increase in BrdU incorporation over medium-only stimulation (B). Pam3Cys (200 ng/ml) and LPS (100 ng/ml) were used as positive controls (B). (A) Shown are representative histograms from three independent experiments. (B) Data are means with SD ($n = 3$) from one of three independent experiments yielding similar findings. The asterisk indicates significant ($p < 0.05$) differences between wild-type LT-IIb-B₅ and S74D mutant.

Fig. 5. Mucosal adjuvanticity of the B pentameric subunit of LT-IIb holotoxin (LT-IIb-B₅)

Groups of BALB/c mice were intranasally immunized with *S. mutans* AgI/II, in the absence or presence of LT-IIb-B₅ or intact LT-IIb adjuvant, at days 1, 15, and 29. Antibody responses (salivary IgA [A], vaginal IgA [B], serum IgA [C], and serum IgG [D]) were monitored at days 22, 36, and 50. Data are means with standard deviations ($n = 6$). Black circles indicate statistically significant ($p < 0.05$) enhancement of antibody responses compared to sham immunization, whereas asterisks show significant ($p < 0.05$) enhancement of antibody responses compared to AgI/II ("Ag") alone.

Table 1Most highly upregulated genes in LT-IIb-B₅-stimulated BMDC*

Gene	LT-IIb-B ₅ -stimulated BMDC		
	wt BALB/c	wt C57BL/6	MyD88 ^{-/-} C57BL/6
Il12a	30.0	11.5	0.6
Il6	19.5	33.9	0.9
Tnf	11.4	30.7	0.9
Ptgs2	11.3	38.0	1.2
Il1b	9.4	13.6	0.6
Il1a	8.2	12.6	1.0
Tlr1	8.2	9.3	1.4
Clec4e	7.8	4.4	1.4
Csf3	4.7	6.7	0.9
Tlr2	3.6	3.2	0.9
Cd80	3.4	3.3	0.8
Cd14	3.4	2.7	0.9
Il10	3.1	4.9	1.2

* Upregulated more than threefold upon 4-h stimulation vs. unstimulated BMDC (TLR Signaling Pathway-focused real-time PCR array; Superarray).