Modulation of Innate and Acquired Immune Responses by *Escherichia coli* Heat-Labile Toxin: Distinct Pro- and Anti-Inflammatory Effects of the Nontoxic AB Complex and the Enzyme Activity[1]

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We have examined the roles of enzyme activity and the nontoxic AB complex of heat-labile toxin (LT) from *Escherichia coli* on its adjuvant and immunomodulatory properties. LTK63, an LT mutant that is completely devoid of enzyme activity, enhanced Th1 responses to coinjected Ags at low adjuvant dose. In contrast, LTR72, a partially detoxified mutant, enhanced Th2 responses and when administered intranasally to mice before infection with *Bordetella pertussis* suppressed Th1 responses and delayed bacterial clearance from the lungs. LTR72 or wild-type LT inhibited Ag-induced IFN-γ production by Th1 cells, and LT enhanced IL-5 production by Th2 cells in vitro. Each of the toxins enhanced B7-1 expression on macrophages, but enhancement of B7-2 expression was dependent on enzyme activity. We also observed distinct effects of the nontoxic AB complex and enzyme activity on inflammatory cytokine production. LT and LTR72 suppressed LPS and IFN-γ induced TNF-α and IL-12 production, but enhanced IL-10 secretion by macrophages in vitro and suppressed IL-12 production in vivo in a murine model of LPS-induced shock. In contrast, LTK63 augmented the production of IL-12 and TNF-α. Furthermore, LTK63 enhanced NF-κB translocation, whereas low doses of LTR72 or LT failed to activate NF-κB, but stimulated cAMP production. Thus, *E. coli* LT appears to be capable of suppressing Th1 responses and enhancing Th2 responses through the modulatory effects of enzyme activity on NF-κB activation and IL-12 production. In contrast, the nontoxic AB complex can stimulate acquired immune responses by activating components of the innate immune system. ***The Journal of Immunology*, 2000, 165: 5750–5759.**

The heat-labile toxin of *Escherichia coli* (LT)[3] has been shown to act as a powerful mucosal adjuvant for nasal or oral delivery of protein Ags (1–3). LT is composed of two subunits, a monomeric enzymatically active A subunit that ADP-ribosylates GTP binding proteins and a pentameric, nontoxic B subunit that binds GM_1 gangliosides at the surface of eukaryotic cells. However, as LT is too toxic for clinical use, nontoxic mutants have been created by site-directed mutagenesis, and it has been reported that these mutants retain certain of the adjuvant properties of LT (4–9). We have previously demonstrated that nasal delivery of an acellular pertussis vaccine with a completely nontoxic mutant, LTK63, enhanced Th1 and Th2 responses, whereas LTR72, which retains 1% of the ADP-ribosyltransferase, selectively enhanced Th2 responses, especially at low adjuvant dose (9).

It has also been reported that LT may inhibit immune response (2), and LT and LT-B have been shown to induce apoptosis in lymphocytes, particularly in $CD8^+$ T cells (10). Cholera toxin (CT), which is closely related to LT, has been shown to inhibit IL-12 production by activated human macrophages and DC and to down-regulate IL-12R expression on activated T cells (11). The proinflammatory cytokine IL-12 is required for the effective priming of Th1 cells in vivo (12); therefore it is an important target for anti-inflammatory therapy (13). It has been reported that a number of pathogens have evolved mechanisms to subvert cellular immune responses by specifically inhibiting this cytokine (13). *Leishmania*-derived phosphoglycan has been shown to inhibit LPS-induced IL-12 synthesis in macrophages (14). During HIV infection CD40L expression on $CD4^+$ T cells is reduced, and this is associated with dysregulated IL-12 production (15). Furthermore, the *Bordetella pertussis* virulence factor, filamentous hemagglutinin (FHA), has been shown to inhibit IL-12 secretion by macrophages (16). Although it has been suggested that both CT and LT inhibit IL-12 production (11), it has also been reported that CT does not affect IL-12 production in LPS and IFN-γ-activated murine macrophages (17). Furthermore, it has been shown that LT-B could inhibit the development of Th1 responses and the development of disease in a murine model of collagen-induced arthritis (18). However, a nontoxic mutant of LT was found to enhance Th1 responses to coadministered Ags (9). Thus, the precise immunomodulatory effects of LT and CT are still not clear, and their mechanisms of action remain to be defined.

The hypothesis that the adjuvant action of LT derives from independent contributions of the A and B subunits (6, 9) may account for some of the apparent discrepancies between studies using

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[3] Abbreviations used in this paper: LT, heat-labile toxin (from *E. coli*); CT, cholera toxin, PT, pertussis toxin; FHA, filamentous hemagglutinin; PRN, pertactin; KLH, keyhole limpet hemocyanin; LN, lymph node; PEC, peritoneal exudate cell; MIP-2, macrophage inflammatory protein-2.

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different LT molecules (4–9, 18, 19). The LT mutants, LTK63, which is completely devoid of enzyme activity and LTR72, which is partially detoxified and can therefore be administered in vivo by several routes (4, 6, 9), have provided us with excellent tools to investigate the role of the enzyme and binding activities of LT on its immunomodulatory function. The present study addressed the hypothesis that the enzyme activity and the nontoxic AB complex of LT have differential immunomodulatory effects on innate immune responses, which result in activation or suppression of the type 1 and type 2 arms of the acquired immune response. We examined the capacity of LT, LTK63, and LTR72 to modulate Th1 and Th2 responses either to injected foreign Ags or to the respiratory pathogen, *B. pertussis*, where Th1 cells play a key role in protection (20, 21). We found that low doses of LTK63 enhanced type 1 and type 2 cytokine production in vivo, whereas LTR72 enhanced Th2 responses, but suppressed Th1 responses and bacterial clearance from the lungs of *B. pertussis*-infected mice. Furthermore, incubation of APC with LTR72 inhibited Ag-induced IFN-γ production by Th1 clones. In contrast, LTR72 and LT enhanced cytokine production by Th2 cells and inhibited LPS-induced IL-12 and TNF-α production by macrophages. Our findings also suggest that the differential pro- and anti-inflammatory actions of the binding and enzyme domains of LT results from their respective effects on NF-κB activation and cAMP production in cells of the innate immune system.

Materials and Methods

Bacterial toxins and Ags

Mutants of LT, LTK63, and LTR72 were created by site-directed mutagenesis as previously described (4, 6). The LT mutants and wild-type LT (referred to as LT hereafter) were found to be free of detectable LPS as determined by the LAL assay (Sigma, St. Louis, MO), with a sensitivity of 15 pg/ml. Heat-killed *B. pertussis* was prepared by incubation of cells at 80°C for 30 min. Purified native pertussis toxin (PT), FHA, and pertactin (PRN) were prepared by Chiron Vaccines (Siena, Italy). Keyhole limpet hemocyanin (KLH) was purchased from Sigma.

Mice

All mice used were female BALB/c, aged 6–8 wk, at the initiation of each of the experiments, obtained from Harlan U.K. (Bicester, Oxon, U.K.), and were housed according to the regulations of the Irish Department of Health.

Parenteral administration of LT mutants

BALB/c mice were injected s.c. into the footpad with 1 μg KLH alone or with 1 μg LTK63 or LTR72 in 25 μl of PBS or PBS only. The draining popliteal lymph nodes (LN) were removed after 7 days and assessed for Ag-induced type 1 or type 2 cytokine production.

Effect of LTK63 and LTR72 on B. pertussis *respiratory infection*

LTK63 and LTR72 (1 μg) were administered to mice by the nasal route 3 days or 3 h before respiratory infection with *B. pertussis*, by resuspending the toxins in 25 μl of PBS and applying them to the external nares with a micropipette following light halothane (Sigma) anesthesia. Mice were challenged with *B. pertussis* by exposure to an aerosol of bacteria for 15 min as previously described (22), and the course of infection was followed by performing CFU counts on lungs at intervals after challenge. Groups of four mice were sacrificed at 0, 7, 14, 21, and 28 days; lungs were removed aseptically and homogenized in 1 ml of sterile physiological saline with 1% casein on ice. Aliquots of 100 μl of undiluted or serially diluted homogenate from individual lungs were spotted in triplicate onto Bordet-Genou agar plates, and the number of colonies was assessed after 5 days of incubation. Results are reported as the mean viable *B. pertussis* for individual lungs from four mice per time point per experimental group. Systemic and local T cell responses were assessed using spleen cells and thoracic LN cells recovered 7, 14, 21, 28, and 35 days after challenge.

Th1/Th2 responses determined by cytokine production

Spleen cells from individual mice or pooled LN cells (2×10^6 cells/ml) were cultured in triplicate at 37°C and 5% CO_2 in RPMI medium (8% FCS) with Ag, either 10^6–10^7 cells/ml heat-killed bacteria or 1–100 μg/ml KLH, medium alone, or 20 ng/ml PMA (Sigma) and 1 μg/ml purified anti-mouse CD3 (PharMingen, San Diego, CA). Supernatants were removed after 72 h and the concentrations of IFN-γ, IL-4, IL-5, IL-10, and IL-12 were determined by immunoassay. Matched pairs of Abs (capture and detection) and the limit of sensitivity of the assays were as follows: 100 pg/ml IFN-γ, R4-6A2, and XMG1.2 (PharMingen); 15 pg/ml IL-4, 11-B-11 (National Institutes of Health, Bethesda, MD) and BVD6-24G2 (PharMingen); 15 pg/ml IL-5, TRFK5, and TRFK4 (PharMingen); 15 pg/ml IL-10, JES5-2A5, and SXC-1 (PharMingen); and 15 pg/ml IL-12 p40, C17.8, and C15.6 (PharMingen). Results are expressed as mean cytokine concentrations, after extrapolation from a standard curve prepared with standard cytokines, for each Ag concentration performed in triplicate on individual spleen cells or pooled LN cells from four or five mice per group.

Effect of LT and LT mutants on Ag presentation and T cell activation

T cell lines were established from mice immunized with a pertussis vaccine by stimulating spleen cells with 1 μg/ml PRN and cloned by limiting dilution as previously described (23). Splenic APC were incubated in petri dishes overnight with 1 μg/ml LT, LTK63, or LTR72 or with medium only. Cells were removed by gently scraping, washed, irradiated (30 Gy), and added to cultures at 2×10^6/ml with 2×10^5 cells/ml PRN-specific Th1 or Th2 clones and 1.0 μg/ml PRN Ag or medium only. T cell activation was assessed by measuring the production of IFN-γ and IL-5 in the culture supernatant after 3 days of culture by specific immunoassay.

Ab assays

Concentrations of serum IgG subclasses specific for PT, FHA, or PRN were determined by ELISA. ELISA plates were coated by incubation overnight with 50 μl of purified Ag (1 μg/ml). After washing and blocking, serially diluted serum samples were added and incubated overnight at 4°C. The bound Abs were detected by alkaline phosphatase-conjugated anti-mouse IgG1 or IgG2a (PharMingen). Results are expressed as end-point titers, calculated by regression of the straight part of a curve of OD vs serum dilution to a cutoff of 2 SD above background control values for serum from naive mice.

Stimulation and inhibition of inflammatory responses in vivo and in vitro

BALB/c mice were injected i.p. or s.c. into the footpad with 1 μg LTK63, LTR72, or LT, and 6 or 24 h later peritoneal exudate cells (PEC) were collected by peritoneal lavage (23), or cell suspensions were prepared from the draining popliteal LN. Inflammatory cytokine production was assessed in lavage fluid and in the draining LN by immunoassays specific for TNF-α, IL-1β, IL-6, IL-10, IL-12 p40, or macrophage inflammatory protein-2 (MIP-2). The matched pairs of mAbs (capture and detection) and the sensitivity of the assays were as follows: 15 pg/ml TNF-α, goat anti-mouse polyclonal Abs (Duoset; R&D Systems, Minneapolis, MN); 15 pg/ml IL-1β, goat anti-mouse polyclonal Abs (Duoset; R&D Systems); 15 pg/ml IL-6, MP5-20F3 and MP5-32C11 (PharMingen); 15 pg/ml IL-10, JES5-2A5 and SXC-1 (PharMingen); 15 pg/ml IL-12 p40, C17.8 and C15.6 (PharMingen); and 60 pg/ml MIP-2, goat anti-mouse polyclonal Abs (R&D Systems). Cell surface expression of MHC class II, B7-1 or B7-2 was assessed by staining with anti-mouse MHC class II, PE-conjugated anti-CD80, or FITC-conjugated anti-CD86 Abs (PharMingen) and analyzed on a Becton Dickinson FACScan flow cytometer (Mountain View, CA) as previously described (24).

To assess the effects of LT and LT mutants on inflammatory cytokine production in vitro the macrophage cell line J774 (American Type Culture Collection, Manassas, VA) were cultured at 1×10^6 cells/ml in complete RPMI medium alone or with 1 μg/ml of LTK63, LTR72, or LT. Supernatants were recovered after 24 h, and the concentrations of TNF-α, IL-6, IL-10, and IL-12 p40 were determined by immunoassay as described above. Alternatively, macrophages were incubated with the toxins for 1 h before stimulation with 1 μg/ml LPS from *E. coli* (serotype 0111:B4; Sigma) and 1 μg/ml murine IFN-γ, a combination known to induce macrophage IL-12 production. Supernatants were removed after 24 h for analysis of cytokine levels. In certain experiments mAbs (10 μg/ml) to IL-10 (JES5-2A5; PharMingen) or TGF-β (A75-3.1; PharMingen) were added with the toxins.

Inhibition of IL-12 production in a murine model of LPS-induced shock

The effects of LT and LT mutants on IL-12 production in vivo were assessed in a murine model of LPS-induced shock (16, 25). BALB/c mice were injected i.p. with 1 μg LT, LTR72, or LTK63 or with PBS only as control, followed 1 h later with 1 μg *E. coli* LPS (serotype 0111:B4; Sigma) by i.v. injection. After 6 h mice were sacrificed, blood was removed from the thoracic cavity, and serum was frozen for cytokine analysis.

cAMP assay

PEC isolated from mice injected i.p. with 1 μg LTK63, LTR72, or LT, 1 μg LPS, or PBS were incubated in complete RPMI on plastic petri dishes at 37°C for 2 h; nonadherent cells were removed; cold RPMI was added; and the adherent cells were removed gently by cell scrapping. The peritoneal macrophages were washed, counted, and resuspended in 0.25 M Tris buffer, pH 8.5, at a concentration of 2×10^6 cells/ml. The levels of cAMP were then determined using a cAMP enzyme immunoassay kit as instructed by the manufacturer (Cayman, Ann Arbor, MI).

NF-κB analysis

NF-κB expression was assessed in peritoneal macrophages isolated from BALB/c mice injected 24 h earlier with LT and LT mutants as described above. Nuclear extracts were prepared from stimulated macrophages and NF-κB expression was measured using an EMSA as previously described (26). Briefly, nonspecific competitor DNA (poly d(I-C)) was added, followed by 0.1 of 10× binding buffer, then the nuclear extract sample and the ^{32}P-labeled DNA fragment probe containing the NF-κB motif (5′-AGT TGA GGG GAC TTT CCC AGG C-3′). The underlined neucleotides are the NF-κB consensus site in the 22-bp oglionucleotide. This mixture was incubated at room temperature for 30 min. After the incubation loading buffer with marker dyes was added, and the samples were loaded onto 4% acrylamide gel (20 × 20 cm), and run at room temperature for 2 h at 150 V. The gel was dried (45 min at 80°C) and autoradiographed overnight, and the bands were visualized and photographed using the Eagle-Eye (Stratagene, La Jolla, CA), and the images were saved using Microsoft Photo-Editor (Microsoft, Redmond, WA).

Results

Differential enhancement of type 1 and type 2 responses in draining LN following coinjection of Ag with LTK63 and LTR72

It has previously been demonstrated that LT or LT mutants can augment immune responses to Ags delivered by the mucosal route (4–9). It has been argued that the adjuvant effect of LT may in part relate to its ability to enhance Ag uptake across mucosal surfaces. However, it appears that LT may have immunomodulatory effects distinct from this activity, and we wished to assess these effects. BALB/c mice were immunized with KLH with or without LTK63 or LTR72 or PBS only (control) into the footpad, and immune responses were evaluated in the local draining LN 7 days later. The in vivo toxicity of LT precluded analysis of the wild-type toxin by this route of administration. LN cells recovered from mice 7 days after injection of KLH secreted low levels of Th2 cytokines following Ag stimulation in vitro. However, LN cells from mice immunized with KLH and 1 μg of LTK63 secreted significant levels of IFN-γ, but there was no significant increase in IL-4 or IL-5 over that observed with KLH alone (Fig. 1). In contrast, the LN cells of mice immunized with KLH and 1 μg LTR72 did not secrete Ag-specific IFN-γ, but did show significantly enhanced production of IL-4 and IL-5. Therefore, 1 μg LTK63 enhances Th1 responses, while the same dose of LTR72 augments a more polarized Th2 response. Consistent with our previous study on nasal delivery (9), we did detect Ag-specific IFN-γ and IL-5 in the draining LN of mice immunized with higher doses of either LT mutant as adjuvants; however, the responses were still biased to Th1 or Th2 with LTK63 or LTR72, respectively (data not shown).

Because IL-12 and IL-10 exert regulatory influences on the induction and activation of Th1 and Th2 cells subtypes, we also examined the production of these cytokines by Ag-activated cells ex vivo. LN cells from mice coinjected 7 days earlier with KLH and 1 μg LTK63 secreted significant levels of IL-12 in response to Ag stimulation in vitro, whereas LTR72 failed to stimulate IL-12 production (Fig. 1). In contrast, amplification of IL-10 production

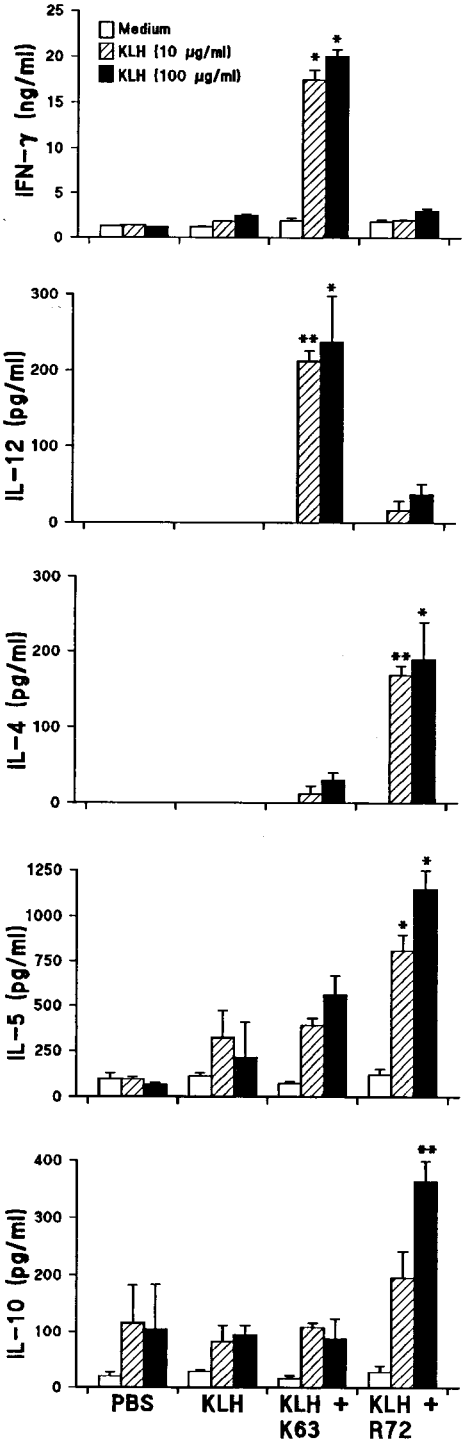

FIGURE 1. Effect of LTK63 or LTR72 on the induction of Th1/Th2 responses in vivo. BALB/c mice were immunized s.c. into hind footpads with 1 μg KLH alone, with 1 μg LTK63 or LTR72, or with PBS only. The popliteal LN cells were isolated 7 days later and stimulated in vitro with 10 or 100 μg KLH or medium only. After 72 h supernatants were removed, and IFN-γ, IL-5, IL-4, IL-10, and IL-12 concentrations were measured by immunoassay. Results are the mean (± SD) cytokine concentrations from triplicate cultures of pooled LN cells. * and **, $p < 0.05$ and $p < 0.01$, respectively, vs KLH alone (by Student's *t* test).

was only observed with LTR72 (Fig. 1). Thus, LTK63 appears to have proinflammatory activity, whereas LTR72 stimulates anti-inflammatory responses in vivo.

Treatment of BALB/c mice with LTR72 before B. pertussis *infection suppresses Ag-specific Th1 responses and IgG2a production and exacerbates infection*

Because LTR72 and LTK63 appear to have differential stimulatory and possibly inhibitory effects on Th1 and Th2 cells when administered by mucosal (9) and parenteral (Fig. 1) routes, we sought to assess their influence on the function of Th1 cells in a murine model of infection, where Th1 cells play an important role in protection. It has been demonstrated that effective clearance of *B. pertussis* from the lungs of infected mice is dependent on acquired immunity mediated by Th1 cells and IgG2a Ab (20, 22) and that the development of protective Th1 cells is enhanced by IL-12 (21). Here, the effects of LTR72 and LTK63 on the immune response and the course of infection were assessed in vivo by intranasal administration of the toxins 3 days before aerosol challenge with *B. pertussis*. There was no significant effect of LTK63 or LTR72 on proliferation (not shown) or of LTK63 on cytokine production by spleen cells recovered 35 days after challenge (Fig. 2). However, there was a significant reduction in *B. pertussis*-specific IFN-γ production by spleen cells from mice treated with LTR72. There was a simultaneous increase in Ag-specific IL-5 (Fig. 2) and IL-4 production (data not shown). Cytokine production by Ag-stimulated cells from the local thoracic LN was consistent with these findings and also demonstrated enhancement of IFN-γ production by LTK63. We observed significant *B. pertussis*-specific IFN-γ production (20–60 ng/ml) by thoracic LN cells from LTK63-treated mice, but low or undetectable levels (<0.1–10 ng/ml) in PBS-treated mice 7, 14, and 21 days after *B. pertussis* challenge. In contrast, IL-4 and IL-5 production was elevated in the LN cells of *B. pertussis* infected mice pretreated with LTR72, but not with LTK63 (data not shown).

An examination of anti-*B. pertussis* IgG subclasses in serum recovered 35 days after *B. pertussis* infection confirmed the suppressive effect of LTR72 on Th1 responses in vivo. Untreated or LTK63-treated *B. pertussis*-infected mice developed Abs predominately of the IgG2a subclass. In contrast, *B. pertussis* infected mice treated with LTR72 had significantly higher levels of IgG1 against the *B. pertussis* Ags PT, FHA, and PRN and had lower levels of IgG2a than mice pretreated with PBS or LTK63 (Fig. 2). Therefore, the administration of LTR72 before infection with *B. pertussis* reversed the IgG2a/IgG1 ratio of *B. pertussis*-specific Ab response.

The effect of LTK63 and LTR72 on the course of infection was assessed by administering the toxin mutants 3 h or 3 days before *B. pertussis* challenge. The course of bacterial clearance was monitored by examining the number of viable bacteria recovered from the lungs of the mice at weekly intervals after challenge. Respiratory infection was significantly exacerbated in mice pretreated with LTR72 (Fig. 3, *A* and *C*). In contrast, there was either no significant difference in the course of infection between LTK63-treated and control mice (Fig. 3*B*) or the CFU values were significantly lower at certain time points after challenge in the LTK63-treated compared with control and/or LTR72-treated mice (Fig. 3*C*).

LTR72 and LT inhibit activation of Th1 cells, and LT enhances activation of Th2 cells in vitro

To determine whether the modulatory effects of LT operate at the level of Ag presentation and T cell activation, studies were performed with Ag-specific T cell clones in vitro. Splenic APC were incubated overnight with LTK63, LTR72, or LT; the cells were then washed, irradiated, and used to stimulate Ag-induced activation of Th1 or Th2 clones specific for PRN from *B. pertussis*. Treatment with LT, LTR72, or LTK63 did not affect the viability of the APC (data not shown). However, pretreatment of APC with LT or LTR72 completely ablated their ability to process or present Ags to Th1 clone, and the difference in the responses from those observed for untreated APC was highly significant ($p < 0.001$; Fig. 4). Although LTK63-treated APC also had reduced ability to support Ag-induced IFN-γ production, this reduction and the level of significance ($p < 0.05$) were not as dramatic as those observed with LT or LTR72. In contrast, Th2 clones stimulated with Ag and

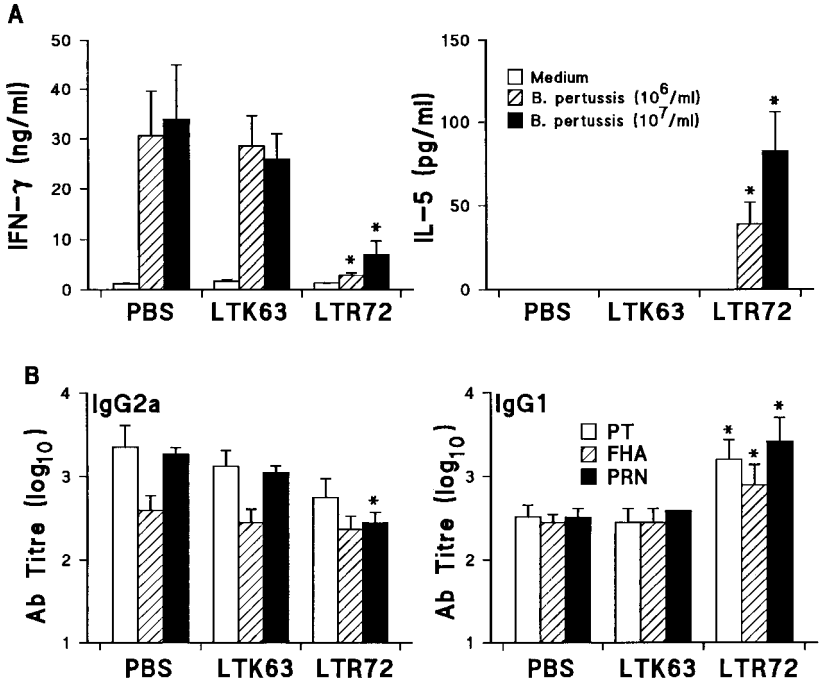

FIGURE 2. Intranasal administration of LTR72 inhibits Ag-specific Th1 and enhances Th2 responses following infection with *B. pertussis*. LTK63, LTR72 (1 μg), or PBS was administered i.n. to BALB/c mice 3 days before aerosol challenge with *B. pertussis*. Spleen and serum samples were isolated 35 days after challenge. *A*, Spleen cells were stimulated in vitro with heat-inactivated *B. pertussis* (10^6 or 10^7 cells/ml) or medium only. Supernatant was removed after 72 h, and IL-5 and IFN-γ concentrations determined by immunoassay. *B*, Serum IgG1 and IgG2a specific for FHA, PT, and PRN were determined by ELISA. Results are the mean ($\pm$ SD) cytokine concentrations or $\log_{10}$ end-point titers for Ab assays for four mice per group tested individually in triplicate. *, $p < 0.05$ vs PBS-treated group (by Student's *t* test).

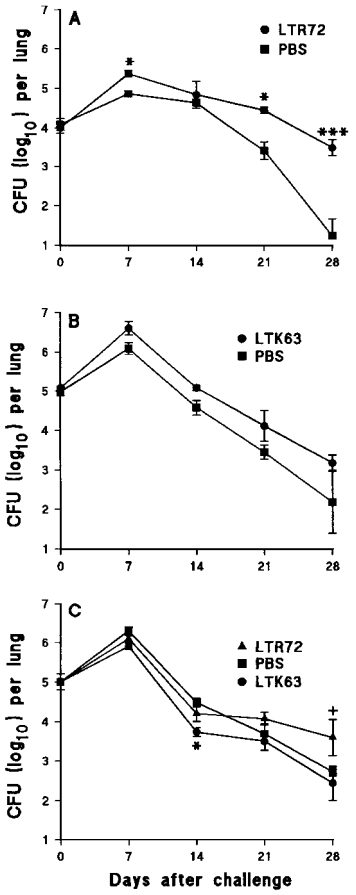

FIGURE 3. Treatment with LTR72 before an infection with *B. pertussis* significantly delays bacterial clearance from the lungs of infected mice. In separate experiments 1.0 μg LTK63 (*A*) or LTR72 (*B*) or PBS only was administered intranasally to groups of 24 BALB/c mice 3 days before infection with *B. pertussis*. *C*, LTK63, LTR72 (1 μg), or PBS was administered intranasally to groups of 24 BALB/c mice 3 h before infection with *B. pertussis*. The course of infection was monitored by assessing the numbers of viable bacteria in the lungs at intervals after challenge. Results are expressed as the mean ($\pm$ SE) lung CFU counts assessed on four individual mice per experimental group at each time point. $*$ and $***$, $p < 0.05$ and $p < 0.001$ vs PBS-treated group; $+$, $p < 0.05$ vs LTK63- or PBS-treated groups (by Student's *t* test).

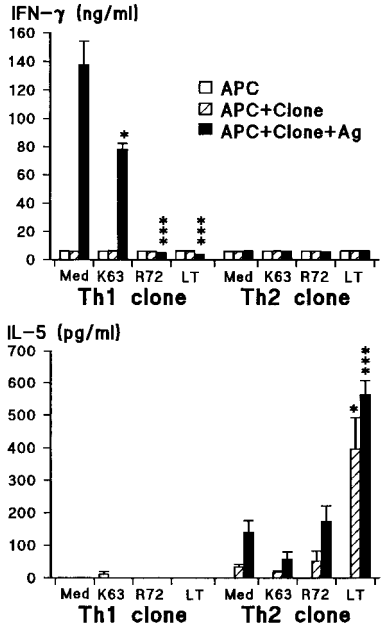

FIGURE 4. Treatment of APC with LTR72 or LT decreases their ability to present Ag to Th1 clones, but enhances activation of Th2 clones. Spleen cells from naive BALB/c mice were cultured for 24 h with LTK63, LTR72, or LT (1 μg/ml) or medium only. The cells were washed, irradiated, and added as 2×10^6 APC to cultures with 2×10^5 PRN-specific Th1 or Th2 clones and 1 μg/ml Ag. Supernatant was removed after 72 h, and IL-5 and IFN-γ were assessed by specific immunoassay. Results are the mean ($\pm$ SD) cytokine concentrations from triplicate cultures. $*$ and $***$, $p < 0.05$ and $p < 0.001$, respectively, vs APC treated with medium alone (by Student's *t* test).

APC pretreated with LT had significantly enhanced IL-5 production (Fig. 4) and proliferation (data not shown). However, this effect was not Ag specific, as the Th2 clones were also activated by the LT-pretreated APC in the absence of added Ag and may reflect a direct effect on T cells due to LT bound to the surface of the APC. Thus, it appears that the binding/enzyme activity of LT activates Th2 cells in a mitogenic fashion.

Recruitment and activation of APC by LT and LT mutants in vivo

Enhancement of inflammatory cytokine and chemokine production and transient recruitment of immune effector cells to the site of immunization are possible mechanisms by which LT may mediate its adjuvant activity. When LT mutants were examined for their ability to stimulate inflammatory cytokine production in vivo, it was found that i.p. or s.c. injection of 1 μg LTK63 and LTR72 significantly ($p < 0.05$ to $p < 0.01$) enhanced local IL-6 and MIP-2 production, whereas LTK63, but not LTR72, also significantly ($p < 0.05$ to $p < 0.01$) enhanced TNF-α and IL-12 production (not shown).

Modulation of costimulatory or MHC class II molecule expression on APC may explain the effects of LT and LT mutants on Ag presentation and T cell activation. BALB/c mice were injected i.p. with LTK63, LTR72, or LT, and 6 h later PEC cells were isolated, and the expression of MHC class II, B7-1 (CD80), and B7-2 (CD86) was examined by flow cytometry. There was no significant difference in MHC class II expression on PEC from mice injected with LTK63, LTR72, LT, or LPS (34.4 $\pm$ 2.89%). However, LTR72 and LT significantly enhanced the percentage of cells expressing B7-1 and LTK63 also consistently, but not significantly, enhanced expression of this costimulatory molecule (Fig. 5). Furthermore, LT significantly enhanced the number of cells expressing B7-2 (Fig. 5).

Suppression of macrophage IL-12 and TNF-α production by LT and LTR72 in vitro

As well as stimulating inflammatory cytokines, it has also been suggested that CT or LT may or may not influence IL-12 production in response to other inflammatory stimuli (11, 17). The modulatory effect of LT or LT mutants on inflammatory cytokine production was examined by assessing their effect on LPS- and IFN-γ-induced production of IL-12, IL-10, IL-6, and TNF-α by the murine macrophage cell line J774. We first examined the stimulatory effect of the LT molecules on macrophage cytokine production in vitro. LTK63 stimulated low, but significant, levels of IL-12 and TNF-α by macrophages ($p < 0.01$ vs unstimulated cells). In contrast, LTR72 or LT induced low levels of IL-10, but failed to

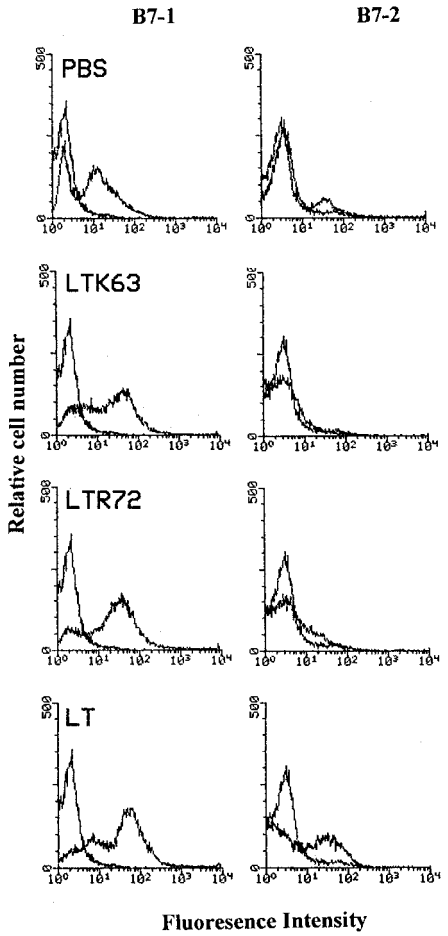

FIGURE 5. LT and LT mutants up-regulate B7 expression. Mice were injected i.p. with LT, LTR72, or LTK63 (1 μg) or with PBS alone. PEC were removed after 24 h and stained with anti-CD80-FITC or anti-CD86-PE and analyzed by flow cytometry. Results are representative FACScan profiles for individual mice from three experiments, with four mice per group in each experiment. *Left profiles* represent fluorescence with an isotype-matched control Ab. The mean ($\pm$ SD) percentage of positive cells and levels of significance vs PBS-treated cells (by Student's *t* test) for B7-1 expression were: PBS, 25.2 $\pm$ 1.2; LTK63, 28.7 $\pm$ 3.4; LTR72, 35.7 $\pm$ 3.0 ($p < 0.05$); and LT, 39.5 $\pm$ 3.8 ($p < 0.01$). For cells expressing B7-2 they were: PBS, 9.2 $\pm$ 1.3; LTK63, 5.0 $\pm$ 1.0; LTR72, 11.2 $\pm$ 3.9; and LT, 20.5 $\pm$ 2.0 ($p < 0.01$).

stimulate TNF-α or IL-12. Both mutants and wild-type toxin stimulated IL-6 production (Fig. 6).

LPS and IFN-γ stimulated IL-12 and high levels of TNF-α production by J774 macrophages, which were completely blocked by coincubation with LTR72 and LT. In contrast, LTK63 either had no effect or a much less significant inhibitory effect (Fig. 6). Furthermore, LT and LTR72, but not LTK63, stimulated high levels of IL-10 in the presence of LPS and IFN-γ. The LT mutants and wild-type LT did not affect LPS and IFN-γ-induced IL-6 production, demonstrating that the modulatory effects on other cytokines did not result from toxicity to the cells. Inclusion of the neutralizing IL-10 Ab partially reversed the inhibitory effect of LTR72 or LT on IL-12 production, but failed to increase the levels of TNF-α produced (Fig. 6). This is consistent with the observation that LT and LTR72, but not LTK63, significantly augmented LPS-induced IL-10 production (Fig. 6). In contrast, an Ab against TGF-β did not alter the modulatory effect of LT or LTR72 on LPS-induced cytokine production (data not shown).

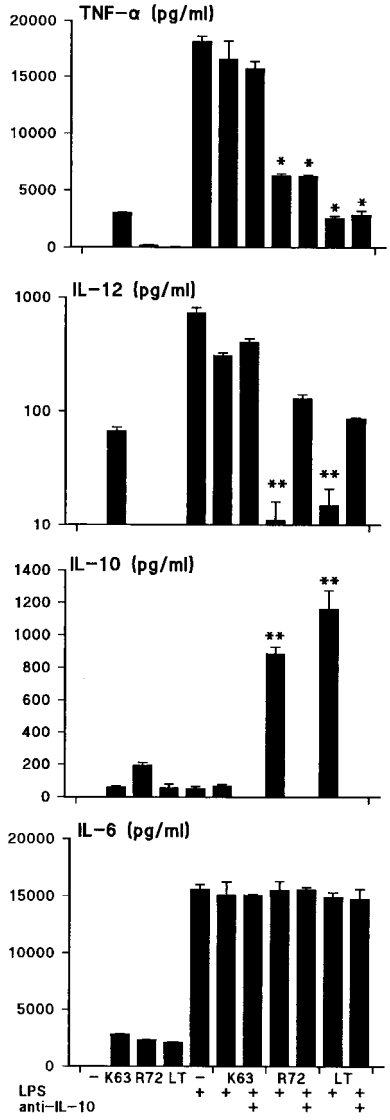

FIGURE 6. Differential effects of LT and LT mutants on pro- and anti-inflammatory cytokine production by macrophages in vitro. J774 macrophages (1×10^6 cells/ml) were stimulated in vitro with LTK63 (K63), LTR72 (R72), or LT (1 μg/ml) alone or with 1 μg/ml LPS and 1 μg IFN-γ, in the presence or the absence of 10 μg/ml of a neutralizing anti-IL-10 Ab. Control cultures included cells incubated with LPS and IFN-γ alone or medium only. After 24 h supernatant was removed, and IL-12, IL-10, IL-6, and TNF-α concentrations were determined by specific immunoassay. Results are the mean ($\pm$ SD) cytokine concentrations from triplicate cultures. * and **, $p < 0.05$ and $p < 0.01$, respectively, vs LPS and IFN-γ only (by Student's *t* test).

LT and LTR72 suppress LPS-induced IL-12 in vivo in a murine model of LPS-induced shock

Having demonstrated that LTR72 and LT have anti-inflammatory activity in vitro, we assessed their inhibitory effects on inflammatory responses in vivo using an established murine model of LPS-induced shock (16, 25). Mice were pretreated with 1 μg PBS, LTK63, LTR72, or LT and were challenged 1 h later with 1 μg *E. coli* LPS, and serum IL-12 concentrations were determined after 6 h. The control mice, pretreated with PBS, had very high levels of serum IL-12 following injection of LPS (Fig. 7). These levels were not significantly different in mice that were pretreated with LTK63. However, the levels of serum IL-12 were significantly

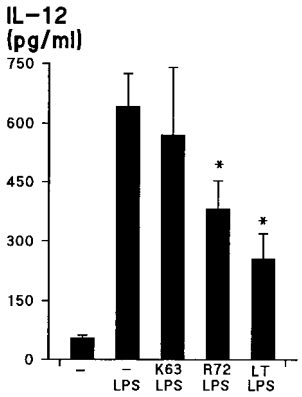

FIGURE 7. LTR72 and LT significantly inhibit IL-12 production in vivo in a murine model of LPS-induced shock. BALB/c mice were injected i.p. with LTK63, LTR72, LT (1 μg), or PBS alone and after 1 h were injected i.v. with 1 μg LPS. Six hours later, blood was removed, and the levels of IL-12 (p40) in the serum were analyzed by immunoassay. Results are expressed as mean ($\pm$ SD) cytokine concentrations from triplicate cultures or serum samples from four mice per group. $*$, $p < 0.05$ vs LPS alone (by Student's *t* test).

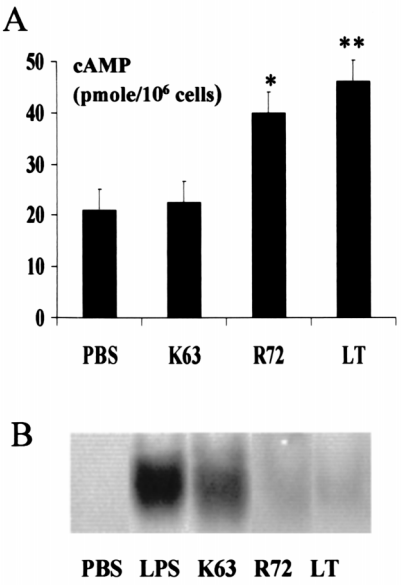

FIGURE 8. LTK63, LTR72, and LT differentially induce cAMP production and NF-κB expression in murine macrophage in vivo. BALB/c mice were injected i.p. with 1 μg LPS, LTK63, LTR72, or LT; or PBS only and after 24 h PEC were removed. *A*, PEC were lysed in Tris-HCl (pH 8.5), and cAMP levels were determined by specific immunoassay. Results are mean ($\pm$ SD) cAMP concentrations from PEC lysates from four mice per group. $*$ and $**$, $p < 0.05$ and $p < 0.01$, respectively, vs PBS (by Student's *t* test). *B*, Nuclear extracts were prepared from the adherent cells, and NF-κB expression was determined by EMSA. The results are representative of three individual mice per group.

reduced in mice pretreated with either LTR72 or LT before challenge with LPS (Fig. 7).

LTK63, LTR72, and LT differentially effect cAMP levels in murine macrophages

The A_1 subunit of LT is an enzyme with ADP-ribosylating activity that binds NAD and transfers the ADP-ribose group to the stimulatory α subunit of G_s, a GTP-binding protein, which regulates the activity of adenylate cyclase (27). Once this G protein is ADP-ribosylated the adenylate cyclase is permanently activated, causing abnormal intracellular accumulation of cAMP (28). Since LTR72 has attenuated enzyme activity (6), and LTK63 is a mutant of LT that is enzymatically inactive (4), their effects on cAMP accumulation may explain their differential influence on IL-12 production and Th1/Th2 induction. BALB/c mice were injected i.p. with different concentrations of LTK63, LTR72, and LT to determine the levels of intracellular cAMP induced in macrophages by the toxins in vivo (Fig. 8*A*). LTK63, the enzymatically inactive toxin, did not activate cAMP production in murine macrophages. However, both LTR72 and LT induced significant cAMP accumulation. The levels of cAMP induced by LT were significantly greater than those induced by LTR72 (Fig. 8*A*).

LTK63, LTR72, and LT differentially affect NFκB expression in murine macrophages

Because the transcription factor NF-κB controls the expression of the genes of many proinflammatory cytokines, including IL-12 and TNF-α, we assessed the effects of LT and LT mutants on the expression of NF-κB. BALB/c mice were injected i.p. with 1 μg LTK63, LTR72, and LT, and the levels of NF-κB expression were assessed in the peritoneal macrophages 24 h later. LTK63 was found to strongly enhance NF-κB expression; the signal with 1 μg was similar to that observed with the same dose of LPS (Fig. 8*B*). In contrast, LTR72 and LT failed to induce NF-κB translocation at the 1-μg dose (Fig. 8*B*), but did so at the higher concentration, but not to the same extent as that observed with LTK63 (data not shown).

Discussion

This study demonstrates that the enzyme and binding activity of LT can differentially affect the production of pro- and anti-inflammatory cytokines and the induction and activation of Th1 and Th2 cells. LTK63, a nontoxic mutant of LT, devoid of enzymatic activity stimulated IL-12 and TNF-α production by macrophages and enhanced Th1 responses to coinjected Ags. In contrast, low doses of LTR72, which retains partial enzymatic activity, suppressed LPS-induced IL-12 production, selectively augmented type 2 responses, and inhibited protective Th1 responses in a murine respiratory infection model. Furthermore, we found that LT and the mutant toxins differentially induce intracellular cAMP accumulation and NF-κB activation in vivo. It appears that the adjuvant activity of LT on Th1 and Th2 responses results from its binding to receptors and activation of NF-κB-transduced events in cells of the innate immune system, whereas the immunomodulatory effect on inflammatory responses, including suppression of IL-12 and TNF-α production, may result from the effect of the enzyme-active domain in elevating intracellular cAMP levels.

The role of the binding and enzyme domains in the adjuvant activity of LT, especially the effect on T cell subtype induction, remains controversial. The majority of studies that have employed nontoxic mutants, including the present investigation, have shown that enzyme activity is not essential for certain of the adjuvant effects of LT (4–9). However, our data reveal that the enzyme activity can exhibit an immunomodulatory effect distinct from adjuvanticity. It has previously been reported that purified or recombinant B subunits of LT or CT are capable of inducing oral tolerance and suppressing inflammatory Th1 responses that mediate autoimmune diseases (2, 18, 29). This is compatible with reports that the B subunit may selectively stimulate the reciprocally regulated Th2 population. However, we observed that the partially toxic mutant LTR72, as well as selectively enhancing Th2 responses to coadministered Ags, was also capable of inhibiting Th1 responses to a potent Th1 cell-inducing respiratory pathogen. In

contrast, the completely nontoxic mutant, which retained receptor binding activity, did not suppress *B. pertussis*-specific IFN-γ production and either had no significant effect or enhanced the rate of clearance of *B. pertussis* from the lungs. Infection of mice (20) or humans (30) with *B. pertussis* results in the selective induction of Th1 cells, and these cells mediate bacterial clearance and confer immunity against subsequent infection. We observed suppression of IFN-γ production, enhancement of IL-4 and IL-5, reversal of the IgG1/IgG2a ratio, and exacerbation of infection in mice treated with LTR72 before respiratory infection with *B. pertussis*. IFN-γ was not elevated in the spleen cells of LTK63-treated mice 35 days after challenge; at this stage the T cell response in the spleen is highly polarized to Th1, and it would be difficult to observe further enhancement with LTK63. However, the Th1 response in the local LN develops late in infection, and we observed augmentation of IFN-γ production in thoracic LN cells from LTK63-treated mice and enhancement of IL-4 and IL-5 in LTR72-treated mice 7–21 days after *B. pertussis* challenge. Thus, in our model system using low doses of toxin, suppression of Th1 responses and enhancement of Th2 responses in vivo is associated with residual enzyme activity of LT.

The differential effects of LTK63 and LTR72 on Ag presentation or activation of Th1 and Th2 cells in vitro support this conclusion. Pretreatment of APC with LT or LTR72 significantly inhibited their ability to stimulate Ag-specific IFN-γ production by a Th1 clone, whereas LT enhanced IL-5 production by a Th2 clone specific for the same unrelated Ag. A role for the enzyme activity in inhibiting APC function is consistent with a report that LT and CT could inhibit the functions of APC by inhibiting intracellular Ag processing in a manner dependent on ADP-ribosyltransferase (31). The treatment of B lymphoma cells with CT was also found to inhibit their APC function by triggering the cAMP cascade, resulting in increased intracellular pH and reduction of the degradation of Ag (32). The enhancement of Th2 cytokine production, which appears to be independent of enzyme activity, may also operate through a nonspecific mitogenic effect of LT on Th2 cells, analogous to the effect of PT on Th1 cells (24). This conclusion is supported by the present study and by the observation that CT-B and CT can dramatically increase Ag-induced IL-4 production from Th2 cells (33).

Transient recruitment of inflammatory cells and up-regulation of MHC or accessory molecules on APC are other mechanisms by which bacterial toxins may enhance Ag presentation and T cell responses to unrelated Ags. We have found that LT or LT mutants significantly enhanced recruitment of cells to LN draining the site of inoculation (E. J. Ryan, E. McNeela, and K. H. G. Mills, unpublished observations). Enhancement of cellular infiltration may be mediated in part by the ability of the toxins to induce the production of IL-6, which has chemotactic properties for lymphocytes (34), and the C-X-C chemokine, MIP-2, the putative functional homologue of human IL-8. LT, LTR72, and LTK63 differentially effected the expression of certain costimulatory molecules on the cells of the innate immune system. Although, we observed little effect on MHC class II expression and marginal up-regulation of CD40L expression on T cells (data not shown), each of the toxins induced modest enhancement of B7-1, whereas wild-type LT and, to a lesser extent, LTR72 enhanced B7-2 production. This finding is supported by a report which demonstrated that enhancement of B7-2 expression on bone marrow-derived macrophages by CT was dependant on cAMP elevation (35). However, it has also been reported that CT enhances B7-1 and B7-2 on B cells, whereas an enzymatically inactive rCTB had no effect (36). Furthermore, Yamamoto et al. (37) showed that native CT or CT mutant E112K enhanced B7-2 expression, but to some extent also enhanced B7-1 on Peyer's patch B cells and macrophages. The observation that enzyme activity is associated with enhanced B7-2 expression is consistent with our data showing selective enhancement of Th2 responses by LT and LTR72 and thus the link between Th2 responses and B7-2 expression (38).

While activation of cytokine secretion and cell surface molecule expression on cells of the innate immune system may explain in part the adjuvant effect of bacterial toxins, it does not explain the suppressive effect of the toxins on inflammatory Th1 responses and immune-mediated diseases. Induction of apoptosis in T cells (2) is a possible explanation for the suppression of Th1 cytokine production. However, cell death would have to be specific for Th1 and not Th2 cells, because LT enhanced proliferation and cytokine production by Th2 clones. At the doses of toxins employed in this study we found no evidence of cell death either in vivo in local LN after injection of LT or LT mutants or in vitro after incubation with APC before stimulation of T cell clones. The induction and activation of Th1 and Th2 cells are differentially regulated by APC type and the cytokine milieu at the site of T cell priming. The macrophage-, dendritic cell-, and neutrophil-derived cytokine IL-12 together with IL-18 produced in response to LPS and other microbial products play a key role in the selective induction of Th1 cells (12). This study demonstrated that LT molecules with complete or partial enzyme activity suppress LPS-induced IL-12 production both in vitro and in vivo. Although it has been reported that LT does not affect IL-12 production (17), albeit using a different experimental system, our results are consistent with those of Braun et al. (11), who showed that both CT and LT could inhibit IL-12 production from activated monocyte-derived DC. Thus, as previously demonstrated for *Leishmania major* (14), HIV (15), measles virus (39), and *B. pertussis* (16), enterotoxigenic *E. coli*, through the ADP-ribosyltransferase activity of its a subunit of LT secreted into the gastrointestinal tract, may have evolved a strategy to subvert protective Th1 responses *in* vivo by inhibiting the production of IL-12.

The distinct modulatory effects of the receptor binding and enzyme activity of LT on Th cell subtypes and inflammatory responses may be related to their respective abilities to enhance or suppress signaling pathways in cells of the innate and acquired immune systems. The B subunit of LT binds to the GM_1 receptor on eukaryotic cells, which leads to the activation of acidic sphingomylinases (40). The activation of these enzymes has been shown to result in the accumulation of intracellular ceramide, which, in turn, can lead to the activation of NF-κB (41), which controls the transcription of a number of genes, such as IL-12, involved in inflammatory responses, or the induction of apoptosis (42, 43). In this study it was shown that the nontoxic mutant LTK63 strongly induces NF-κB activation in murine macrophage cells in vivo and in vitro. It is possible that binding of the B subunit to GM_1 stimulates NF-κB activation, leading to enhancement of proinflammatory cytokine production and cell surface molecule expression on cells of the innate immune system.

In contrast, the ADP-ribosyltransferase activity of the A subunit of LT leads to the accumulation of intracellular cAMP; LTR72 and LT enhanced intracellular cAMP, whereas the enzyme-inactive mutant LTK63 had no effect. It has previously been shown that agents that up-regulate cAMP also block proliferation and cytokine secretion by Th1 cells and activate Th2 cells (43, 44). cAMP inhibits expression of the IL-2 gene through NF-AT sites in T cells (45) and elevates the transcription factor GATA-3, which regulates IL-5 gene expression (46). Thus, the inhibitory effect of LTR72 and LT on Th1 cells may be mediated through the induction of cAMP stimulated by the enzyme activity of LT. It has also been reported that cAMP has inhibitory effects on the production of

inflammatory cytokines by macrophages (47), and this may in part explain the function of the enzyme activity in suppression of IL-12 production. We found that LTR72 and LT enhanced cAMP and suppressed LPS-induced IL-12 and TNF-α production in murine macrophages, whereas LTK63, a nontoxic mutant of LT, did not cause any increase in cAMP levels and had only a small, but not significant, effect on LPS-induced IL-12 production in vitro and in vivo. Furthermore, LTR72 and LT significantly enhanced LPS-induced IL-10 production, and a neutralizing Ab to IL-10 partially attenuated the inhibitory effect on IL-12 production.

It appears that the enzyme and binding domain both contribute to the immunomodulatory effects of LT, and certain of the apparently conflicting reports in the literature may reflect the Ags used, the dose of adjuvant, the route of delivery, and the immune responses examined to document an adjuvant effect. It has been reported that the adjuvant effect for Ab responses is minimal with the B subunit, intermediate for nontoxic mutants, and maximal for partially toxic or the wild-type toxin and increases with dose of the toxin, except for LT at doses above 10 μg, reputedly due to its toxicity (6). We have observed that the modulatory effect of the LT and LT mutants on T cell responses is also dose dependent, but is more complex and involves differential effects on Th1 and Th2 responses (9). The nontoxic mutant LTK63 enhanced Th1 responses at 1 μg and both Th1 and Th2 responses at 10 μg. In contrast, LTR72, which retains residual toxicity, selectively enhanced Th2 responses, but suppressed IL-12 production and Th1 responses at the lower doses and had a modest enhancing effect on both T cell subtypes at the higher dose. The immunomodulatory activity of LT appears to be mediated both by a direct effect on T cells and by inhibitory and stimulatory influences of the enzyme and binding domains on cells of the innate immune system, which, in turn, differentially influence the induction of Th1 and Th2 cells.

It has been shown that elevated cAMP levels can inhibit NF-κB-mediated transcription through competition between cAMP response element binding protein and p65 for limiting amounts of the transcriptional coactivator CCREB binding protein (47). Therefore, the enzyme activity of the A subunit may have an inhibitory effect on the NF-κB activation induced by the binding of the B subunit. We found that LTK63 strongly enhanced NF-κB expression in macrophages even at relatively low doses, whereas LTR72 and LT only stimulated NF-κB at high concentrations and not to the same extent as LTK63. It appears that the very low levels of enzyme activity are sufficient to suppress the toxin's ability to induce NF-κB, but at a high dose this inhibitory effect is partially overcome by other activities of the AB complex on NF-κB activation or perhaps by binding to other receptors, which may mediate effects through distinct signaling pathways. In conclusion, our findings provide evidence that the adjuvant effect of LT is derived from independent contributions of the A and B subunits, and that the pro- and anti-inflammatory effects of LT appears to be dependent on the balance between the two distinct biochemical signaling pathways inhibited or activated by the nontoxic AB complex and enzyme activity, respectively.

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