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J Immunol 2011; 186:5896-5906; Prepublished online 13 April 2011; doi: 10.4049/jimmunol.1003789
http://www.jimmunol.org/content/186/10/5896

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**Escherichia coli** Heat-Labile Enterotoxin Promotes Protective Th17 Responses against Infection by Driving Innate IL-1 and IL-23 Production

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*Escherichia coli* heat-labile enterotoxin (LT) is a powerful mucosal adjuvant; however, it is associated with toxic effects when delivered intranasally, and its mechanism of action is poorly understood. In this article, we demonstrate that LT acts as a highly effective adjuvant when administered parenterally, promoting Ag-specific IL-17, as well as IFN-γ, IL-4, and IL-10 production in response to coadministered Ags. We found that the adjuvant activity of LT was mediated in part by inducing dendritic cell (DC) activation; LT promoted CD80 and CD86 expression by DCs and enhanced IL-1α, IL-1β, and IL-23 production. An LT mutant, LTK63, that lacks enzyme activity was less effective than the wild-type toxin in promoting DC maturation and the development of Ag-specific Th17 cells. LT enhanced IL-23 and IL-1α production by DCs via activation of ERK MAPK and IL-1β secretion through activation of caspase-1 and the NLRP3 inflammasome. These cytokines played a major role in promoting Th17 responses by LT and LTK63 as adjuvants was significantly reduced in IL-17–defective mice. Our findings provide clear evidence that LT can promote protective immune responses in part through induction of innate IL-1 and, consequently, Th17 cells. The Journal of Immunology, 2011, 186: 5896–5906.

Heat-labile enterotoxin (LT) is a key immunomodulatory molecule produced by *Escherichia coli* and one of the most potent adjuvants available, especially for immunization via mucosal routes (1). However, its use as an intranasally delivered adjuvant in humans has been compromised by the adverse effects observed in certain recipients of nasal influenza vaccines formulated with LT or an LT mutant as adjuvant (2, 3). LT has also been shown to act as an adjuvant when administered via parenteral routes and was demonstrated to be safe in humans when delivered transcutaneously in a clinical trial of an influenza virus vaccine (4).

LT is a member of the AB class of bacterial toxins, composed of an enzymatically active A subunit with ADP-ribosyltransferase activity and a B subunit that mediates binding of the molecule to eukaryotic cell surfaces (5). LT increases the concentration of intracellular cAMP via activation of adenylate cyclase. The induction of cAMP by LT is considered a dominant factor in mediating its adjuvant effects (6, 7). However, studies in animal models using the partially active derivative, LTR72, and the enzymatically inactive mutant LTK63, have shown that these molecules retain at least some of the adjuvant properties of LT (8–14). These findings clearly indicate that the immunomodulatory properties associated with LT are not entirely dependent on ADP-ribosyltransferase activity.

Many immunomodulatory molecules from pathogens have evolved mechanisms for evading host immune responses by subverting innate and consequently adaptive immune responses. Interestingly, certain pathogen-derived immunomodulatory molecules can also selectively promote adaptive immunity by activating APCs, and this is thought to be the major basis of their adjuvant function. Dendritic cells (DCs) are the most potent APC capable of stimulating naïve T cell differentiation and are a key target for many immunomodulatory molecules and adjuvants. Cholera toxin (CT), another AB enterotoxin, which has considerable homology with LT, enhances costimulatory molecule expression on murine DCs (6, 15, 16). In addition, LT has been shown to selectively upregulate the expression of CD80, but not CD86, on DCs (17) and murine macrophages (18). Neither CT nor LT alone induces the production of cytokines by DCs, although CT induced the production of the chemokine MIP-2 (19). Both CT and LT have been shown to modulate cytokine production in response to TLR agonists. The toxins suppressed IL-12 and TNF-α production by human monocytes and DCs, or murine macrophages in response to a variety of stimuli, including LPS and CD40L (6, 18, 20). CT also inhibited LPS-induced MIP-1α and MIP-1β production by murine DCs, whereas it enhanced LPS-induced IL-10, IL-1β, and IL-6 production.
Although it is well established that LT is a potent immunomodulatory molecule and acts as a strong adjuvant, capable of promoting the induction of Th1 and Th2 responses, the possibility that LT also promotes the induction of Th17 cells has not been examined. Therefore, a key aim of this study was to investigate the ability of LT to promote the induction of Th17 cells and to examine the mechanism underlying its adjuvant activity, in particular its ability to activate signaling pathways in DCs that mediate production of key cytokines that enhance the development of Th17 cells.

We show that LT promotes Ag-specific IL-17, as well as IFN-γ, IL-5, and IL-10 production and IgG Abs to coadministered Ag when delivered by a parenteral route. The adjuvanticity of LT was not entirely due to the enhancement of intracellular cAMP, as the enzymatically inactive derivative LTK63 also promoted Ag-specific T cell responses, although not to the same extent as LT. Both LT and LTK63 were capable of inducing DC maturation, with upregulation of CD80 and CD86 costimulator molecule expression. Furthermore, LT and LTK63 synergized with LPS to induce IL-1β and IL-23 secretion by DCs, which in turn promoted Ag-specific IL-17 and IFN-γ production by CD4+ T cells. IL-10 and IL-23 production by DCs in response to LT and LPS was dependent on signaling via ERK MAPK, whereas IL-1β production was mediated via activation of nucleotide-binding domain and leucine-rich repeat-containing protein 3 (NLRP3) inflammasome and caspase-1. Finally, using knockout mice and an infection model, we demonstrate that the induction of IL-1β by innate immune cells and consequently IL-17-producing T cells plays a key role in the adjuvant activity of LT and its ability to promote protective immune responses in vivo.

Materials and Methods

Mice

BALB/c and C57BL/6 mice were purchased from Harlan UK. BALB/c mice expressing a transgene for the DO 11.10 TCR specific for aa 323–339 of OVA and I-Aδ, NLRP3-defective (Nlrp3−/−), IL-1R type I-defective (Il1r1−/−) and IL-17A-defective (Il17a−/−) mice were bred on site. Experiments were performed under license from the Department of Health with the approval of the Trinity College Dublin BioResources Ethics Committee.

Toxin and derivatives

Wild-type (WT) LT and the site-directed mutant LTK63 (Ser63Lys) were constructed and purified as described (21). LTK63 has previously been shown to have undetectable ADP-ribosylation activity, with polyarginine as the substrate (21). The endotoxin content of LT and LTK63 was <0.1 pg LPS per microgram of toxin.

Immunizations

BALB/c, C57BL/6, Il17a−/−, or Il1r1−/− mice were immunized i.p. or s.c. with keyhole limpet hemocyanin (KLH) or experimental acellular pertussis vaccine (Pa) formulated with LT or LTK63 as the adjuvant. Mice were immunized once with KLH (20 μg/mouse) formulated with LT or LTK63 as the adjuvant. Mice were euthanized mice. Immature DCs were cultured from single-cell suspensions of bone marrow in RPMI 1640 medium supplemented with 20 ng/ml GM-CSF in the form of supernatant from a GM-CSF–expressing J558 cell line. After 3 d incubation, 20 ml fresh medium, containing 20 ng/ml GM-CSF, was added to each culture flask. Loosely adherent cells were obtained on day 6 and recultured in the presence of 20 ng/ml GM-CSF. On day 10, the loosely adherent cells were harvested and DCs were cultured in tissue culture plates. Prior to stimulation, DCs were allowed to rest overnight in medium containing 10 ng/ml GM-CSF.

For DC adoptive transfer, DCs were cultured with medium alone, KLH (20 μg/ml), or KLH with LT or LTK63 (1 μg/ml) for 24 h. Cells were washed twice in PBS and injected i.c. into the footpads of naive mice (5 × 10⁵/mouse). Mice were sacrificed 7 d later for assessment of immune responses.

Innate cytokine production induced by LT and LTK63 in vitro and in vivo

DCs were stimulated with LT (1 μg/ml), LTK63 (1 μg/ml), LPS (100 ng/ml; Alexis), LPS and LT or LTK63, or with medium only. After 6 or 24 h, supernatants were removed and the concentrations of IL-1α, IL-23, and IL-6 were quantified by ELISA. For assessment of IL-12p70 production, DCs were preincubated with LT for 1 h or LTK63 for 6 h before the addition of LPS (100 ng/ml). Alternatively, DCs were stimulated for 3 h with LT or LTK63 (1 μg/ml) or medium only, before being cocultured with CD4 T cells stimulated with anti-CD3 (2 μg/ml) and anti-CD28 (2 μg/ml). After 24 h, supernatants were removed and tested for IL-1β, IL-10, and IL-23 production by ELISA. To examine in vivo production of IL-1 and IL-23, mice were injected in the footpads with LT, LTK63 (10 μg/mouse), or PBS. The draining popliteal lymph nodes were removed after 2 and 6 h and homogenized, and the clarified supernatant was used to quantify production of IL-1β, IL-10, and IL-23 by ELISA.

Cell stimulation with LT- or LTK63-activated DCs

DCs (5 × 10³ cells/ml) from BALB/c mice were incubated for 24 h with OVA323-339 peptide (10 μg/ml; New England Peptide) alone, with LPS (100 ng/ml), or with LT or LTK63 (1 μg/ml), or were pretreated with LT (1 h) or LTK63 (6 h) prior to the addition of LPS. DCs were then washed and cocultured with purified DO 11.10 CD4+ T cells (CD4 Subset Column Kit; R&D) at a ratio of 4:1, with or without IL-1Ra (10 μg/ml), anti–IL-23 (10 μg/ml; eBioscience), and anti–IL-6 (100 ng/ml; R&D). After a further 72 h, supernatants were removed and tested for IL-17 production by ELISA. Alternatively, CD4 T cells from Bordetella pertussis–infected mice were cultured in the presence of DCs that had been stimulated with LT (1 μg/ml) or medium only and then washed before coculture with the T cells. After 72 h of coculture, supernatants were removed and IL-17A (hereafter referred to as IL-17) and IL-17F production was quantified by ELISA.

Serum Ab titers

The levels of KLH-specific IgG Abs in sera of immunized mice were determined by ELISA. Microtiter plates were coated with KLH (5 μg/ml) and blocked (10% milk), and the Ab responses were detected using biotin-conjugated rat anti-mouse IgG mAb, IgG2a, or IgG1 (Caltag). Results are expressed as log₂ endpoint Ab titers.

Flow cytometry

DCs were surface stained for CD11c, CD80, and CD86 (all from BD Biosciences). Spleen or lymph node cells were stimulated with KLH and after 5 d restimulated with PMA (10 ng/ml; Sigma-Aldrich) and ionomycin (1 μg/ml; Sigma-Aldrich) for 6 h in the presence of brefeldin A (5 μg/ml) for the final 5 h. Cells were then blocked with Fcγ block (1 μg/ml; eBioscience), fixed, and permeabilized (Fix&Perm; ADG), followed by surface staining for CD3 and CD4 (eBioscience) and intracellular staining for IL-17 or IFN-γ (both from eBioscience). FACScan analysis was performed on a CyAn ADP flow cytometer (DakoCytomation), which was calibrated using BD Comp Beads (BD Biosciences). Results were analyzed using Summit (DakoCytomation) or FlowJo (Tree Star) software.

Real-time quantitative PCR

Total RNA was extracted from DCs or spleen cells, using the TRIzol/ chloroform method. Isolated RNA was transcribed into cDNA, using a high-capacity CDNA reverse transcription kit (ABI). Transcripts were quantified by real-time quantitative PCR on an ABI 7500 Fast Real-Time PCR System with Applied Biosystems predesigned Taqman Gene Expression Assays and reagents. For each sample, mRNA was normalized to 18S rRNA and is expressed as fold difference compared with cells incubated in medium only.
IL-1β protein was detected in supernatants of stimulated DCs. Whole protein in supernatants was extracted using the methanol/chloroform method. ERK MAPK phosphorylation was assessed in LT- or LTK63-activated DC cell lysates. Samples were then boiled (95–99°C) for 5 min and loaded onto an SDS-PAGE gel (15%) with m.w. markers (Precision Plus Protein Standards; Bio-Rad). Proteins were transferred to nitrocellulose membranes using a wet transfer system. Transferred membranes were blocked using blocking buffer (5% [w/v] nonfat dried milk [Marvel] in 1× PBS Tween) for 1 h, washed, and incubated at 4°C overnight with anti-IL-1β (0.1 μg/ml; R&D) or anti-p-ERK (0.1 μg/ml; Santa Cruz Biotechnology). Membranes were washed and incubated with specified secondary Ab (0.1 μg/ml; Sigma-Aldrich) for 1 h. Blots were developed by chemiluminescence (LiteAblot; Euroclone).

B. pertussis infection
Mice were infected with B. pertussis (strain Tohama I; 338) by aerosol challenge. The course of B. pertussis infection was followed by performing CFU counts on lungs from individual mice. Lungs of infected mice were harvested 2 h, 5 d, and 10 d postinfection, into 1% casein salt solution and homogenized. Then 100 μl undiluted or serially diluted homogenate was plated in triplicate onto Bordet–Gengou agar plates, and the number of CFU was estimated after 5 d of incubation at 37°C. Bacterial loads are represented as log10 bacteria per mouse lung.

Statistical analysis
Data were compared by one-way ANOVA. Where significant differences were found, the Tukey–Kramer multiple comparisons test was used to identify differences between individual groups.

Results
LT is a potent parenterally delivered adjuvant for Th17 responses
LT acts as an adjuvant when coadministered with Ag via various routes (1, 22). Mucosal delivery of LT has been shown to induce a mixed Th1/Th2-type response, as well as IgG and IgA Ab production. However, the ability of LT to promote Th17 cells following parenteral immunization has not been investigated. This study examined the adjuvant effect of LT delivered by s.c. injection and assessed the role of the enzyme activity of LT, using the nontoxic mutant LTK63, previously shown to lack ADP-ribosylation activity (21). BALB/c mice were immunized s.c. in the footpad with PBS alone, KLH, or KLH with LT or LTK63, and the cellular immune responses were assessed 7 d later.

Immunization of mice with KLH alone did not induce any cytokine production from lymph node cells following Ag stimulation ex vivo. In contrast, immunization of mice with KLH in combination with LT or LTK63 promoted Ag-specific IL-17, IFN-γ, IL-10 (Fig. 1A), and IL-5 (data not shown) production. At equivalent doses (1 μg per mouse), LT was significantly more effective than LTK63 in promoting KLH-specific IL-17 production, but IFN-γ concentrations were similar with a dose of 1 μg for either toxin. However, when mice were immunized with KLH in the presence of a 10-fold higher dose of LTK63 (10 μg/mouse), the concentration of IL-17 was comparable to that induced by the lower dose of LT (1 μg per mouse) (Fig. 1B). These data suggest that the enzyme activity of LT plays a significant role in the induction of Th17 cells, but not Th1 responses.

The induction of Th17 cells was confirmed by intracellular cytokine staining; immunization of mice with KLH in the presence of LT or LTK63 induced IL-17–secreting CD4+ T cells in the spleen (Fig. 1C). We also assessed the induction of IL-17– and IFN-γ–secreting cells in lymph nodes from immunized mice and found that both LT and LTK63 promoted induction of T cells that secreted IL-17 or IFN-γ, but not both cytokines (Fig. 1D). These findings demonstrate that LT and LTK63 promote the induction of distinct populations of Th1 and Th17 cells.

LT and LTK63 also promoted robust KLH-specific IgG, IgG1, and IgG2a Ab production, although LTK63 induced lower Ab titers when compared with LT (Fig. 1E). These data demonstrate that LT is a powerful adjuvant for Ags delivered by parenteral route immunization. Because it has previously been shown that LTK63

FIGURE 1. Parenteral immunization with LT promotes Th17 cell responses. A, Mice (n = 5) were injected s.c. with PBS, KLH (20 μg/mouse), or KLH with LT (1 μg/mouse) or LTK63 (1 μg/mouse). After 7 d, lymph node cells (1 × 106 cells/ml) were restimulated in vitro with KLH (2–50 μg/ml) or with medium alone. Supernatants were recovered after 72 h, and IL-17, IFN-γ, and IL-10 concentrations were quantified by ELISA. B–D, Mice were immunized and responses tested as in A, except mice received 10 μg/mouse KLH and 10 μg/mouse LTK63. B, Responses were examined as in A. C, Spleen cells (1 × 106 cells) were restimulated with medium or KLH (25 μg/ml). After 5 d, cells were restimulated for 5 h with PMA (10 ng/ml), ionomycin (1 μg/ml), and brefeldin A (5 μg/ml). Cells were washed, blocked, and surface stained for CD3 and CD4, as well as stained for IL-17 intracellularly. Percentages refer to CD3+ CD4+ IL-17+ T cells. D, Lymph node cells (1 × 106 cells) were restimulated and assessed as described in C and stained for intracellular IL-17 and IFN-γ. E, Results are representative dot plots from 5 mice per group. F, Mice were immunized as in A, and serum samples were analyzed for KLH-specific IgG, IgG1, and IgG2a by ELISA. Results are representative of four experiments. *p < 0.05, **p < 0.01, ***p < 0.001, versus KLH alone.
lacks ADP-ribosylation activity (21), our findings suggest that induction of cAMP plays an important role, especially for Th17 responses. However, we show, importantly, that not all adjuvant activity associated with LT is dependent on its enzyme activity.

**LT and LTK63 modulate the activation of DCs and their ability to drive Ag-specific T cell responses**

As DCs play a critical part in directing T cell responses, we examined the role of DCs in mediating the adjuvant effects of LT and LTK63. DCs were pulsed with KLH alone or in the presence of LT or LTK63 in vitro for 24 h and transferred s.c. to naive mice. Ag-specific T cell responses were assessed 7 d later, by stimulating lymph node cells with Ag ex vivo.

DCs pulsed with Ag alone induced low levels of KLH-specific IFN-γ and IL-10, but no IL-17. Transfer of DCs pulsed with KLH in the presence of LT promoted the induction of KLH-specific IL-17, IFN-γ, and IL-10 (Fig. 2). Transfer of DCs pulsed with KLH and LTK63 induced comparable levels of IFN-γ, but did not increase KLH-specific IL-17 to the same extent as observed with LT. Transfer of KLH-pulsed DCs activated with LTK63 induced a stronger IL-10 response than that induced with LT. This observation is consistent with our findings that LT and LTK63, which lacks enzyme activity (21), enhance TLR-induced innate IL-10 via activation of p38 MAPK (C.F. Brereton and K.H.G. Mills, unpublished observations). Furthermore, transfer of DCs primed with LT and Ag induced stronger IFN-γ production than that induced with DC stimulated with LTK63. Because IFN-γ can limit IL-10 production (23), this may explain the stronger IL-10 responses with LTK63-activated DCs. Collectively, our data demonstrate that LT and LTK63 can act directly on DCs to enhance their capacity to promote T cell responses in vivo. Our results provide further evidence that the enzyme activity of LT plays an important role in promoting Th17 responses.

**FIGURE 2.** Both LT and LTK63 enhance the ability of DCs to promote Ag-specific T cell responses in vivo. DCs were stimulated for 24 h with medium only, KLH (20 μg/ml), or KLH with LT or LTK63 (1 μg/ml). Cells were washed and transferred s.c. to naive mice. After 7 d, lymph node cells from individual mice (1 × 10⁶ cells/ml) were restimulated with KLH (2–50 μg/ml) or with medium alone. Supernatants were recovered after 72 h, and IL-17, IFN-γ, and IL-10 concentrations were quantified by ELISA. Results are representative of three experiments. **p < 0.01, ***p < 0.001, versus KLH alone.

**LT and LTK63 upregulate costimulatory molecules on DCs and can modulate TLR-ligand–induced cytokine responses**

Mature DCs express high levels of costimulatory molecules, including CD80 and CD86, and expression of these molecules is one of the key signals that enable DCs to activate naïve T cells. To examine the effects of LT and LTK63 on DC maturation, DCs were cultured for 24 h with LT or LTK63, or with medium alone, and analyzed for surface expression of costimulatory molecules. Incubation of DCs with LT or LTK63 enhanced CD80 and CD86 expression on CD11c⁺ cells (Fig. 3A). Although the level of enhancement of CD80 and CD86 by both toxins was statistically significant, the enhancement of costimulatory molecule expression was more pronounced with LT than with LTK63 (Fig. 3A). These data demonstrate that upregulation of costimulatory molecules by LT is not totally dependent on its enzyme activity.

We next examined the ability of LT to promote cytokine and chemokine production and to modulate TLR-induced cytokine production by DCs. We have previously shown that LT enhances LPS-induced IL-10 and suppresses IL-12 production by DCs (18). In the current study, we focused particularly on cytokines involved in the differentiation and expansion of Th17 cells. Unlike LPS, LT or LTK63 alone did not induce IL-1α, IL-1β, IL-23, or IL-6 expression (Fig. 3B, C). However, LT and LTK63 did induce MIP-2 production by DCs (Fig. 3D). Furthermore, LT was capable of synergizing with LPS to enhance IL-1β, IL-1α, and IL-23 production (Fig. 3B). LTK63 also significantly enhanced LPS-induced IL-1α, IL-1β, and IL-23 protein expression, but the concentrations were lower than those induced with LT (Fig. 3B). LT and LTK63 did not augment LPS-induced IL-6 production (Fig. 3B), but both LT and LTK63 did synergize with LPS to promote IL-6 mRNA expression (Fig. 3C). In contrast, LT and LTK63 suppressed LPS-induced IL-12p70 production (Fig. 3D). Suppression of LPS-induced IL-12p70 was observed following 1-h preincubation (Fig. 3D) or coincubation (not shown) with LT, but required a 6-h preincubation with LTK63 (Fig. 3D). LT and LTK63 did not induce cell death in DCs, as evidenced by LDH release (data not shown). These findings demonstrate that LT, and to a lesser extent, LTK63 are capable of enhancing TLR-induced cytokines known to be involved in promoting the induction and expansion of Th17 cells.

**LT induces IL-1 and IL-23 production in vivo and following coculture with activated T cells in vitro**

Despite the fact that LT and LTK63 alone acted as adjuvants to promote T cell responses in vivo and in DC transfer experiments, they require a second signal from a TLR agonist to promote innate cytokine production in vitro. It is possible that the second signal in vivo is provided by endogenous TLR agonists or by activated T cells. Therefore, we examined the capacity of LT or LTK63 to induce innate cytokine production in vivo. We found that injection of mice with LT or LTK63 alone, without an added TLR agonist, induced the production of IL-1β, IL-1α, and IL-23 in the draining lymph node 2–6 h later (Fig. 4A). We next examined the possibility that T cells may provide a second signal for innate cytokine production by DCs in vitro. DCs were stimulated with LT or LTK63 in the presence or absence of activated T cells. The results revealed that coincubation of LT- or LTK63-activated DCs with T cells activated with anti-CD3 and anti-CD28 promoted the secretion of IL-1β and IL-1α (Fig. 4B). DCs also produced IL-23 in response to LT when cocultured with activated T cells. In contrast, T cells cultured with LT or LTK63 in the absence of DCs did not produce IL-1β, IL-1α, or IL-23 (data not shown). Collectively, these findings indicate that LT can not only enhance Th17-
promoting innate cytokines in response to TLR agonists in vitro but also can induce these cytokines in vivo without exogenous TLR agonists, and suggest that a second signal may be provided in vivo by pathogen-associated molecular patterns, damage-associated molecular patterns, or activated T cells.

**LT and LTK63 induce IL-23p19 expression via the ERK MAPK pathway**

Having established that LT enhanced IL-23 production by DCs, we examined the signaling pathways involved. A number of studies have demonstrated that components of the MAPK pathway play a major part in the regulation of DCs and macrophages that promote Th1/Th2 cell differentiation (24–26). In particular, the MAPK ERK pathway has been shown to have an important function in regulating IL-12 production (27) and in mediating TLR-induced IL-23 production by DCs (28).

Both LT and LTK63 promoted phosphorylation of ERK1 and ERK2 (p44/p42) in DCs, which was detectable after 10 min and sustained for at least 12 h (Fig. 5A). We next examined the role of ERK in the induction of cytokine expression by LT and LTK63, using the MEK1/2 inhibitor UO126. The enhancement of LPS-induced IL-23 and IL-1α expression by LT and LTK63 was moderately inhibited by the addition of UO126 (Fig. 5B). LT augmented IL-23p19 mRNA ~30-fold over the level of IL-23p19 mRNA expressed in unstimulated DCs (Fig. 5C). Inhibition of ERK suppressed the induction of IL-23p19 expression by LT. LTK63 induced a 10-fold increase in IL-23p19 mRNA expression, which was completely inhibited by UO126 (Fig. 5C). These findings demonstrate that LT promotes IL-23 and IL-1α induction in part via activation of ERK MAPK.
BLOT analysis.

After 24 h, supernatants were collected for analysis of IL-1 \( \beta \) production by ELISA. * \( p < 0.05 \), ** \( p < 0.001 \) versus medium, \( + \) with versus without UO126.

**FIGURE 5.** LT- and LTK63-enhanced IL-23p19 and IL-1\( \beta \) secretion is dependent on ERK MAPK signaling. A, DCs (1 \( \times 10^6 \) cells/ml) were stimulated with LT or LTK63 (1 \( \mu \)g/ml) for between 10 min and 48 h. Cell lysates were probed for p-ERK or \( \beta \)-actin as a loading control by Western blot analysis. B, DCs (5 \( \times 10^5 \) cells/ml) were pretreated with the ERK inhibitor UO126 (5 \( \mu \)M) for 30 min prior to incubation with LT or LTK63 (both 1 \( \mu \)g/ml) for 15 min, followed by stimulation with LPS (100 ng/ml). After 24 h, supernatants were collected for analysis of IL-1\( \alpha \) and IL-23 production by ELISA. * \( p < 0.05 \), ** \( p < 0.001 \) versus medium, \( + \) with versus without UO126. C, DCs (1 \( \times 10^6 \) cells/ml) were incubated with UO126 (5 \( \mu \)M) for 30 min prior to the addition of LT or LTK63 (1 \( \mu \)g/ml) or with medium only. RNA was isolated from bone marrow-derived DCs 12 h later and \( IL23a \) gene expression determined by real-time PCR. The data represent the fold change for \( IL23a \) mRNA expression relative to cells incubated in medium only, following normalization to the endogenous control, 18S RNA. Results are representative of three experiments. * \( p < 0.05 \), ** \( p < 0.001 \) versus medium, \( + \) with versus without UO126.

**LT induces mature IL-1\( \beta \) secretion from DCs via activation of caspase-1 and NLRP3**

Because IL-1\( \beta \) and inflammasome activation have been shown to play an important role in the induction of Th17 cells (29–31), we examined the signaling pathways involved in the induction of IL-1\( \beta \) production by LT. IL-1\( \beta \) is initially transcribed in a proform that requires cleavage by the cysteine protease, caspase-1, for activation. Activation of caspase-1 also requires cleavage from a proform by a multiprotein complex termed an inflammasome. Inflammasomes are composed of an NLRP, which complexes with apoptosis-associated speck-like protein cleaves procaspase into its active form.

We investigated whether LT and LTK63 promote active IL-1\( \beta \) secretion. DCs were incubated with medium, LPS alone, or LPS with LT or LTK63. Western blot analysis showed that LPS, but not LT or LTK63, induced pro–IL-1\( \beta \), which was enhanced by addition of LT. LPS also induced a small amount of mature IL-1\( \beta \), which was greatly enhanced in the presence of either LT or LTK63 after 18 h of incubation (Fig. 6A). As a positive control, DCs were incubated with ATP and LPS or with medium alone for 1 h, which has previously been shown to induce active IL-1\( \beta \) secretion (32). LPS, in the presence of ATP, induced a band at 17 kDa after 1 h, indicative of mature IL-1\( \beta \) production (Fig. 6A). Analysis of IL-1\( \beta \) at the mRNA level revealed that LPS (but not LT or LTK63 alone) induced pro–IL-1\( \beta \), which was enhanced by addition of LT or LTK63 (Fig 6\( B \)), suggesting that LT enhances synthesis as well as processing of IL-1\( \beta \) by LPS-primed DCs.

Having shown that LT and LTK63 induced the secretion of active IL-1\( \beta \) by LPS-primed DCs, we examined the role of caspase-1 and NLRP3 in this process. Blocking caspase-1 with the inhibitor YVAD-fmk suppressed LT- and LTK63-induced IL-1\( \beta \) production in LPS-primed DCs (Fig. 6C). In contrast, the caspase-1 inhibitor had no effect on IL-1\( \alpha \) production. Addition of exogenous KCl, which blocks inflammasome activation and caspase-1 cleavage by inhibiting K\(^+\) ion transport, also suppressed LT-induced IL-1\( \beta \) (Fig. 6C). Moreover, the enhancement of LPS-induced IL-1\( \alpha \) and IL-1\( \beta \) production by LTK63 was significantly reduced when DCs were incubated with KCl (Fig. 6C). In addition, LT-induced IL-1\( \beta \) production in LPS-primed DCs was significantly reduced in DCs derived from \( Nlrp3^{-/-} \) mice (Fig. 6D). These findings demonstrate that LT induces IL-1\( \beta \) production in part through activation of caspase-1 and the NLRP3 inflammasome complex.

**IL-1 signaling plays a crucial role in the capacity of LT and LTK63 to promote Ag-specific IL-17 production by CD4 T cells in vitro and in vivo**

We have demonstrated that LT and LTK63 enhance IL-1\( \alpha \), IL-1\( \beta \), and IL-23 production by DCs and IL-6 mRNA expression, crucial cytokines for Th17 cell development. Furthermore, we have shown that LT and LTK63 promote Th17 and Th1 responses following coadministration with a foreign Ag or by transfer of activated Ag-pulsed DCs. Therefore, we examined the role of IL-1\( \alpha \), IL-23, and IL-6 in the induction of Ag-specific T cell responses by LT and LTK63. DCs were stimulated with OVA peptide alone or in the presence of LPS, LT, LTK63, or with LT or LTK63 prior to the addition of LPS. After 24 h, DCs were washed and cocultured with purified OVA-specific CD4\(^+\) T cells from DO 11.10 mice in the presence or absence of IL-1Ra, anti-IL-23, or anti–IL-6. After 72 h, supernatants were removed and tested for IL-17 and IFN-\( \gamma \) production.

CD4\(^+\) T cells cocultured with DCs pulsed with medium only did not produce IL-17 or IFN-\( \gamma \). DCs pulsed with OVA peptide induced low concentrations of IFN-\( \gamma \) and IL-17 production by CD4\(^+\) T cells (Fig. 7). Stimulation of DCs with LT or LTK63 enhanced OVA-specific IL-17 production but did not augment IFN-\( \gamma \) production. Treatment of DCs with LPS enhanced OVA-specific IL-17 and IFN-\( \gamma \) (Fig. 7). Coincubation of DCs with LT significantly augmented the enhancing effect of LPS on IL-17 but suppressed IFN-\( \gamma \) production, whereas addition of LTK63 did not significantly alter the effect of LPS-activated DCs on IL-17 or IFN-\( \gamma \) production. The results indicate that IFN-\( \gamma \) induced in CD4\(^+\) T cells following incubation with DCs pulsed with OVA and LTK63 in the presence of LPS is stronger than that induced with DCs pulsed with OVA and LT. One explanation is that LT is a more potent inhibitor of LPS-induced IL-12p70; this is consistent with the demonstration that under certain conditions LT, but
NLRP3 or Supernatants were removed after 6 h and tested for IL-1β, followed by stimulation with LPS (100 ng/ml) or medium alone. Production by ELISA. Results are representative of two to four experiments. ***

B. DCs were incubated for 30 min with LT or LTK63 (1 μg/ml) in the presence and absence of LPS (100 ng/ml) or medium alone. Cells were isolated in TRizol, and Il1b gene expression was examined by real-time PCR. ***p < 0.001 versus medium; **p < 0.01, ***p < 0.001 versus LPS only. C. DCs were pretreated with the caspase-1 inhibitor (30 μM) or KCl (50 μM) for 30 min prior to incubation with LT or LTK63 (1 μg/ml) for 1 h, followed by stimulation with LPS (100 ng/ml) or medium alone. Supernatants were removed after 6 h and tested for IL-1β or IL-1β production by ELISA. ***p < 0.001 versus medium. D. DCs from C57BL/6 or Nlpr3−/− mice were incubated with LT, LPS, or LT + LPS, or with medium alone. Supernatants were removed after 6 h and tested for IL-1β production by ELISA. Results are representative of two to four experiments. ***p < 0.001 versus LPS, +++p < 0.001 versus WT. Med, medium.

not LTK63, can suppress Th1 responses either by inhibiting IFN-γ production from T cells or indirectly through modulation of APCs (33).

The addition of IL-1Ra almost completely inhibited IL-17 induced by DCs stimulated with LT or LTK63 alone or with LPS (Fig. 7). Anti–IL-23 also significantly reduced IL-17 production induced by DCs stimulated with LPS and LT or LPS and LTK63, but not with LT or LTK63 alone. In contrast, anti–IL-6 had little effect on IL-17 production. IL-1Ra also suppressed IFN-γ induced in response to LPS or LPS and LTK63. These findings demonstrate a crucial role for IL-1 in the immunopotentiating activity of LT and LTK63, especially for Th17 cells, and suggest that the induction of IL-23 also functions in the induction of Th17 responses by LT combined with a TLR agonist in vitro.

The role of LT-induced IL-1 in driving Th17 responses in vitro was confirmed using CD4+ T cells from Il1r1−/− and WT mice infected with B. pertussis as the source of memory CD4+ T cells. CD4+ T cells from WT or Il1r1−/− mice cultured with control DCs incubated with medium only did not produce IL-17 (Fig. 8). In contrast, CD4+ T cells from WT mice cocultured with DCs stimulated with LT induced significant concentrations of IL-17 and IL-17F (Fig. 8). IL-17 and IL-17F secretion was significantly lower using CD4+ T cells purified from Il1r1−/− mice. LT-activated DCs did not promote IFN-γ production by CD4+ T cells in vitro, and the low concentrations of IFN-γ produced by CD4+ T cells cocultured with unstimulated DCs was reduced when the DCs had been stimulated with LT (data not shown). This observation is consistent with the inhibitory effect of LT on IL-12 production in vitro.

Having shown a crucial role for IL-1 in the induction of Th17 cell responses by LT in vitro, we next examined whether IL-1 was also required for the ability of LT and LTK63 to modulate T cell responses in vivo. C57BL/6 and Il1r1−/− mice were immunized s.c. with KLH alone or in the presence of LT or LTK63. After 7 d, lymph node cells were restimulated ex vivo and examined for cytokine production. Immunization of mice with KLH and LT or LTK63 induced strong IL-17, IFN-γ, and IL-10 production (Fig. 9). The Ag-specific IL-17 and IFN-γ responses promoted by LT or LTK63 were abrogated in Il1r1−/− mice; however, there was no effect on Ag-specific IL-10 production. These data establish a crucial role for IL-1 in the ability of LT and LTK63 to promote the induction of Th17 and Th1 cells.
Immunization with pertussis Ags and LT as an adjuvant promotes Ag-specific IL-17 responses, which confer protection against B. pertussis infection

Once we demonstrated that LT promotes induction of Ag-specific Th17 responses in vitro and in vivo, we applied this finding to a model of infection, to address the hypothesis that LT may act as an adjuvant for protective immunity to a pathogen, at least in part by promoting Th17 cell responses. WT C57BL/6 or Il17−/− mice were immunized i.p. with an experimental Pa (genetically detoxified pertussis toxin and FHA) in combination with LT and boosted 28 d later; WT and Il17−/− mice were immunized with PBS only as a positive control. To examine the nature of the immune response prior to infection, spleen cells from vaccinated WT mice were harvested and restimulated ex vivo on day 41. WT mice immunized with PBS or Pa alone failed to induce Ag-specific cytokine or chemokine responses. In contrast, mice immunized with Pa in the presence of LT induced Ag-specific IL-17, IL-17F, IFN-γ, IL-4, IL-21, IL-10, CCL20, and CXCL2 (Fig. 10A, 10B).

Two weeks after the second immunization, WT and Il17−/− mice were challenged with B. pertussis. Nonimmunized control C57BL/6 mice developed a typical B. pertussis infection after aerosol challenge, with a sustained bacterial load at d 5 post-infection, followed by modest reduction in CFU at d 10 (Fig. 10C). Control nonimmunized Il17−/− mice had a higher bacterial burden than WT mice, which is consistent with the role of IL-17 in natural and whole-cell vaccine–induced immunity to B. pertussis (31, 34). Immunization of WT mice, with Pa and LT as the adjuvant, significantly reduced the bacterial burden in the lungs of infected mice at day 5, with almost undetectable CFU by day 10. In contrast, Il17−/− mice immunized with Pa and LT had a significantly higher bacterial burden on day 5 and day 10, which was ~100-fold greater than that observed in immunized WT mice (Fig. 10C). An examination of local cytokine production in the lungs 5 d after B. pertussis challenge revealed that production of IL-17, IL-17F, IFN-γ, IL-4, and IL-13 was enhanced in mice immunized with Pa and LT when compared with mice immunized with PBS (Fig. 10D). As expected, IL-17 was undetectable in Il17−/− mice, but all other cytokines examined were produced at similar concentrations in Il17−/− and WT mice (Fig. 10D). These findings indicate that immunization with B. pertussis Ags, with LT as ad-

Discussion

To our knowledge, this study has demonstrated for the first time that LT enhances the induction of innate cytokines by DCs that promote the development of Th17 cells, which play a major role in protective immunity against bacterial infection. We showed that LT promoted DC maturation and enhanced TLR agonist–induced IL-1β production through activation of caspase-1 and the NLRP3 inflammasome complex. LT also enhanced LPS-induced IL-1α and IL-23 expression through activation of ERK MAPK in DCs. These innate cytokines promote the development of Th17 cells in vitro and in vivo.

The present study reveals that LT and LTK63 induced the expression of IL-23p19 mRNA in DCs and enhanced LPS-induced IL-1α, IL-1β, and IL-23 production by DCs. Although neither LT nor LTK63 alone induces IL-12p40 mRNA expression, activation of DCs with TLR agonists induces IL-12p40 mRNA expression (C.F. Brereton and K.H.G. Mills, unpublished observations). This observation may explain how LT in combination with LPS is capable of enhancing IL-23 protein secretion; it is consistent with a report that PGE2 primes DCs for LPS-induced IL-23 (35). Of interest, LT induces IL-23, as well as IL-1β and IL-1α, protein expression in vivo in draining lymph nodes after infection into the footpad, and in vitro by DCs when coadministered with activated T cells. The latter may provide the second stimulus

**FIGURE 8.** LT-activated DCs promote IL-17 production by memory CD4+ T cells in an IL-1–dependent fashion. CD4+ T cells from WT or Il1r1−/− mice infected with B. pertussis were cultured with DCs that had been stimulated for 6 h with LT (1 μg/ml). DCs were washed before coculture with T cells. After 72 h, coculture supernatants were removed, and IL-17 and IL-17F production was quantified by ELISA. ***p < 0.001 versus T cell- and medium-incubated DCs, **p < 0.001 versus WT.

**FIGURE 9.** The adjuvant activity of LT and LTK63 for induction of Ag-specific Th17 and Th1 cells in vivo is dependent on IL-1 signaling. C57BL/6 (WT) and Il1r1−/− (KO) mice were immunized s.c. with either KLH (10 μg/mouse) alone or in the presence of LT (1 μg/mouse) or LTK63 (10 μg/mouse). On day 7, lymph node cells (1 × 10⁶ cells/ml) from individual mice were harvested and restimulated ex vivo with KLH (10 or 50 μg/ml) or with medium alone. After 72 h, supernatants were recovered and tested for IL-17, IFN-γ, and IL-10 production by ELISA. Results are representative of three experiments. *p < 0.05, **p < 0.01, ***p < 0.001 versus KLH only, **p < 0.001 versus WT.**
representative of two experiments. **

The endogenous control, 18S RNA. ***

PCR relative to cells incubated in medium only, following normalization to versus PBS, ###

challenge of lungs from naive D. pertussis

mice were challenged with LT (1 0.01, ***

p 0.001 versus naive, +

n 0.05, ++/

mice were immunized i.p. with

Parenteral immunization with B. pertussis Ags and LT as adjuvant promotes Ag-specific Th17 cells, which are protective against B. pertussis infection. C57BL/6 and II17−/− mice were immunized i.p. with Pa (5 μg of PT9K/129G and 5 μg FHA per mouse) alone or in combination with LT (1 μg/mouse) or with PBS alone and boosted after 28 d. A and B, Spleen cells (2 × 10⁶ cells/ml) were harvested from the mice 13 d postboost (n = 4) and restimulated in vitro with medium or FHA (4 μg/ml) for 72 h. A. Supernatants were removed and tested for IL-17 and IL-

Ags and LT as mucosal route (1, 38), we found that parenteral immunization of mice with Ag in the presence of LT or LTK63 promoted the induction of Ag-specific IFN-γ and IL-17, as well as IL-5, in vivo. The induction of Th1 responses is at variance with the in vitro data showing that LT suppresses IL-12 production by DCs (Ref. 18 and the current study). However, we found that IFN-γ production by T cells induced in vitro by DCs activated with LT or LTK63 and LPS and in vivo by immunization with LT and Ag was significantly reduced in the absence of IL-1 signaling. This finding suggests that IL-10 or IL-1β, rather than IL-12, may be the critical cytokine through which LT promotes the induction of Th1 as well as Th17 cells. Furthermore IL-17, but not IFN-γ, production by Ag-specific T cells stimulated with Ag-pulsed and LT-activated DCs was also suppressed by neutralizing IL-23, but not by neutralizing IL-6. This observation is consistent with our previous reports that IL-23 synergizes with IL-1α or IL-1β to drive IL-17 production by CD4+ Th17 cells, as well as by γγ T cells (29, 39).

A number of recent reports have highlighted the importance of the inflammasome complex in the immunopotentiating activity of different adjuvants, including nanoparticles (40) and alum (41), although the latter is still controversial (42). It has also been established that the inflammasome plays a key role in promoting IL-17-producing T cells during infection and in inflammatory disorders (30, 31). The stimuli that drive the assembly of the inflammasome remain poorly understood, but K+ efflux is thought to be an important initiating event, at least for NLRP1- and NLRP3-containing complexes (43, 44). We found that both LT- and LTK63-induced IL-1β production was dependent on activation of caspase-1 and K+ efflux. Furthermore, DCs from Nlrp3−/− mice failed to induce IL-1β production in response to LT in LPS-primed DCs. These findings demonstrate that induction of IL-1β through activation of caspase-1 and the NLRP3 inflammasome complex is a key mechanism by which LT acts as an adjuvant.

In addition to the role of IL-1β, our results suggest that induction of IL-1α and IL-23 is also involved in the adjuvant function of LT. IL-1α, like IL-1β, can synergize with IL-23 to promote IL-17 production by T cells but does not require processing by caspase-1. We have previously reported that activation of ERK MAPK signaling plays a role in TLR-induced IL-23 and IL-1β production and subsequent Th17 cell differentiation (28). In the present study, we found that both LT and LTK63 induced sustained activation of ERK in DCs and that inhibition of ERK suppressed LT- and LTK63-induced IL-23p19 mRNA, as well as IL-23 and IL-1α protein production by LPS-primed DCs.

Our study clearly shows that LT mediates its adjuvant activity by activating signaling pathways in DCs that lead to production of the inflammatory cytokines IL-1α, IL-1β, and IL-23, which promote the development of Th17 cells. Furthermore, our data indicate that LT promotes Th17 responses by enhancing IL-1 pro-
duction via activation of the NLRP3 inflammasome and enhancing IL-23 production in DCs through activation of the ERK MAPK pathway and that this was largely mediated by the enzyme activity of the toxin. The induction of IL-10–secreting Th2 or Th1–

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References


