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MHC Class I-Restricted Cytotoxic Lymphocyte Responses Induced by Enterotoxin-Based Mucosal Adjuvants

Cameron P. Simmons,* Pietro Mastroeni,* Ray Fowler,* Marjan Ghaem-maghami,* Nils Lycke,† Mariagrazia Pizza,† Rino Rappuoli,† and Gordon Dougan*†

The ability of enterotoxin-based mucosal adjuvants to induce CD8+ MHC class I-restricted CTL responses to a codelivered bystander Ag was examined. *Escherichia coli* heat-labile toxin (LT), or derivatives of LT carrying mutations in the A subunit (LTR72, LTK63), were tested in parallel with cholera toxin (CT) or a fusion protein consisting of the A1 subunit of CT fused to the Ig binding domain of *Staphylococcus aureus* protein A (called CTA1-DD). Intranasal (i.n.) immunization of C57BL/6 mice with CT, CTA1-DD, LT, LTR72, LTK63, but not rLT-B, elicited MHC class I-restricted CD8+ T cell responses to coadministered OVA or the OVA CTL peptide SIINFEKL (OVA257–264). CT, LT, and LTR72 also induced CTL responses to OVA after s.c. or oral coimmunization whereas LTK63 only activated responses after s.c. coimmunization. rLT-B was unable to adjuvant CTL responses to OVA or OVA257–264 administered by any route. Mice treated with an anti-CD4 mAb to deplete CD4+ T cells mounted significant OVA-specific CTL responses after i.n. coadministration of LT with OVA or OVA257–264. Both 51Cr release assays and IFN-γ enzyme-linked immunospot assays indicated that IFN-γ−/− and IL-12 p40−/− gene knockout mice developed CTL responses equivalent to those detected in normal C57BL/6 mice. The results highlight the versatility of toxin-based adjuvants and suggest that LT potentiates CTL responses independently of IL-12 and IFN-γ and probably by a mechanism unrelated to cross-priming. *The Journal of Immunology,* 1999, 163: 6502–6510.

In experimental animals, cholera toxin (CT) and *Escherichia coli* heat-labile enterotoxin (LT) are recognized as potent mucosal adjuvants (1, 2). Unfortunately, in humans, oral ingestion of microgram quantities of either LT or CT is sufficient to induce severe diarrhea. The generation of nontoxic LT and CT derivatives that retain adjuvant activity could provide a safer route to the evaluation of these toxins as mucosal adjuvants in humans. Both LT and CT are composed of distinct A and B subunits. The pentameric B subunit (LT-B) contains five identical polypeptides and targets glycosphingolipid receptors on the surface of eukaryotic cells. The A subunit (LT-A) is tightly associated with LT-B and is an ADP-ribosyltransferase, an enzymatic activity responsible for toxicity. The molecular structure of LT has served as a template to generate mutant derivatives, which retain adjuvanticity but have reduced or undetectable toxicity (3). For example, substitution of Ala72 to Arg (LTR72) or Ser 63 to Lys (LTK63) in LT-A generates LT derivatives with adjuvant activity but with significantly reduced (LTR72) (4) or undetectable (LTK63) (5) enzymatic activity and toxicity. Mutagenesis of the A subunit of CT (CT-A) has similarly yielded attenuated CT derivatives which retain adjuvant activity (6, 7). In another approach, the CT-A1 subunit has been expressed attached to a different targeting domain, the Ig binding region of *Staphylococcus aureus* protein A. The resultant fusion polypeptide, CTA1-DD, targets Abs and B cells in vitro and has mucosal and systemic adjuvant activity following intranasal (i.n.) and parenteral administration, respectively (8).

LTR72 and LTK63 can adjuvant systemic and local humoral immune responses to coadministered Ags after vaginal, i.n., or oral immunization (4, 5). However, individual LT derivatives are associated with different levels of adjuvant activity. For example, LTR72 is as effective as LT in inducing Ab production by a bystander Ags (4). LTK63 has reduced adjuvant activity, compared with LTR72 but is significantly more active than LT-B, which is a poor mucosal adjuvant (9). The usefulness of attenuated toxins based on LT and CT is highlighted by their capacity to elicit protective immune responses to Ags from *Helicobacter pylori* (10), *Salmonella dublin* (11), *Streptococcus pneumoniae* (12), and *Candida albicans* (13).

CTLs play a critical role in controlling many infections and may enhance the success of a vaccine against mucosally transmitted pathogens (14). Relatively little work has been performed on the ability of LT and CT derivatives to prime CTLs. Bowen et al. (15) reported that oral or i.v. coadministration of CT with OVA-primed mice for OVA-specific CTL. CT has also been used to generate CTLs against a HIV peptide and a peptide from OVA (16). LT and LTK63 were similarly shown to adjuvant CTL responses to a coadministered class I binding peptide after i.n. immunization (17). In this paper we compare the potential of several different derivatives of ADP-ribosylating toxins as mucosal adjuvants for CD8+ CTL induction. OVA, and the immunodominant H-2b-restricted class I binding epitope from OVA (OVA257–264), were used as model bystander Ags. The contribution of CD4+ T cells and the type 1 cytokines IL-12 and IFN-γ to OVA-specific CTL induction

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3. Abbreviations used in this paper: CT, cholera toxin; LT, *E. coli* heat-labile toxin; i.n., intranasal; sCLN, superficial cervical lymph nodes; ELISPOT, enzyme-linked immunospot; SCF, spot-forming cell.
Materials and Methods

Cells lines and media

Myeloplasma-free EL4 cells, which only express MHC class I (18), were maintained in RPMI 1640 containing 10% FCS (Sigma, St. Louis, MO). 5 × 10^3 M 2-ME, 2 mM L-glutamine (Sigma), 100 U penicillin/ml (Sigma), and 100 μg/ml streptomycin (cRPML) (Sigma). EG7OVA, a subclone of EL4, stably transfected with the gene encoding OVA (18) (kind gift from A. Mowat, University of Glasgow, Glasgow, U.K.) was maintained in cRPML containing 200 μg/ml G418 (Life Technologies, Paisley, Scotland). All cells were cultured in humidified air with 5% CO2 at 37°C.

Ags and Abs

OVA (grade V) and wild-type CT were purchased from Sigma. Wild-type porcine LT, LTK63, LTR72, and rLT-B were purified as described (3). CTA1-Ags and Abs were purchased from PharMingen. Briefly, nitrocellulose-based 96-well microtiter plates (Multiscreen-HA, Millipore, Hertfordshire, U.K.) were coated overnight with 50 μl/well of either 4 μg/ml anti-IFN-γ (R46A2) or 4 μg/ml anti-IL-4 (BVD4-1D11) Ab diluted in carbonate buffer (pH 9.6). After washing three times with filtered PBS, all wells were blocked with 200 μl of cRPML to 2–3 h at 37°C. Following removal of the blocking medium, spleen cells from individual mice were added to the wells in duplicate (maximum 5 × 10^5 cells/well in 200 μl), serially diluted, and cultured for 20 h at 37°C. 5 % CO2 in the presence or absence of 1 μM OVA257–264 peptide. Cells were removed by washing three times with PBS, followed by a further three times with PBS and Tween 20 (0.05% v/v); then 50 μl of the biotinylated anti-IFN-γ (XMG1.2) or anti-IL-4 (BVD6-24G2) Abs (1 μg/ml in filtered PBS and Tween 20) was added to each well for 2 h. After washing plates five times with filtered PBS and Tween 20, a 1/1000 dilution of Extravidin-alkaline phosphatase (Sigma) was added to all wells for 1–2 h at room temperature. Finally, after washing three times with PBS and Tween 20 and once with PBS alone, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Fast BCIP/NBT; Sigma) dissolved in 10 ml of distilled water was added as substrate. Spots, representing single IFN-γ or IL-4-producing cells, were counted using a dissecting microscope. The number of peptide-specific spot-forming cells (SFCs) was determined by subtracting the number of spots obtained with PBS stimulated with medium from those stimulated with OVA257–264. Depletion of CD4+ T cells

C57BL/6 mice, 6–8 wk old, were depleted of CD4+ T cells by i.p. administration of 0.5 mg GK1.5 Ab on day 2 and day 4 before primary immunization; depletion was maintained by administering 0.5 mg Ab on day 5, 10, and 15 after primary immunization. Control mice were administered 0.5 mg rat IgG (Sigma) on the same days. The efficiency of depletion was assessed by staining spleen and CLN cells, with the noncompeting FITC-labeled anti-CD4 Ab (clone RM4-4) (PharMingen). Appropriate FITC-labeled isotype-control Abs were used in all experiments. Stained cells were analyzed on a FACSort flow cytometer (Becton Dickinson, San Diego, CA).

Statistical analysis

Mean IFN-γ ELISPOT numbers were compared using the nonparametric Mann-Whitney t test.

Results

ADP-ribosylating toxins adjuvant OVA-specific CTL responses after i.n. immunization

Previous studies showed that LT and CT can adjuvant CTL responses to i.n. coadministered peptides (16, 23). To establish whether this observation extended to i.n. coadministered whole proteins and to facilitate a direct comparison of LT and CT as

Restimulation and 51Cr release assays

Spleens and superficial cervical lymph nodes (sCLN) were aseptically removed from mice that had been killed by cervical dislocation or exsanguinated under terminal anesthesia. Single cell suspensions from pooled sCLNs (n = 3), or pooled spleens (n = 3), were prepared by passing organs through 100-μm nylon sieves (Marathon Laboratories, London, U.K.). After lysis of splenic erythrocytes (U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD), spleen cells were treated with 3.5 × 10^5 leukocytes from the spleen or sCLN were cultured for 6 days in upright T25 tissue culture flasks in 10 ml of cRPML in the presence of 3 × 10^6 gamma-irradiated (20,000 rads) E.G7-OVA cells. Graded numbers of effector cells, consisting of cells that remained viable after the culture period, were harvested and cocultured in triplicate with 5,000 or 10,000 Na^1CrO_4-labeled EL4 or E.G7-OVA target cells for 4–5 h at the indicated E:T ratios in 200 μl cRPML. A total of 100 μl of culture supernatant was then removed into 100 μl of OptiPhase “Hisafe” scintillation mixture (Fisher Chemicals, Leicester, U.K.), and the amount of Na^1CrO_4 was measured using a 1450 Microbeta liquid scintillation counter (Wallac, Milton Keynes, U.K.). The percentage killing was calculated using the following formula: [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100, where spontaneous release represents the counts obtained when the target cells were lysed with 1% Triton X-100. Each experiment was repeated at least twice.

Enzyme-linked immunospot (ELISPOT) assays

The ELISPOT assays were performed as previously described (22) and all Abs were purchased from PharMingen. Briefly, nitrocellulose-based 96-well microtiter plates (Multiscreen-HA, Millipore, Hertfordshire, U.K.) were blocked by addition of 1/1000 in PBS and Tween 20 containing 0.2% (w/v) BSA for 1 h. Plates were washed twice with PBS and Tween 20 before sera from individual mice was added and serially diluted in PBS and Tween 20 containing 0.2% (w/v) BSA and incubated for 2 h at 37°C. The wells were washed with PBS and Tween 20 before addition of 100 μl of rabbit anti-mouse IgG-HRP conjugate (Dako, Buckinghamshire, U.K.) diluted 1/1000 in PBS and Tween 20 containing 0.2% (w/v) BSA at 37°C. Finally, after washing with PBS and Tween 20, Ag-bound Ab was detected by addition of α-phenylenediamine substrate (Sigma) and the absorbance read. Titers were determined arbitrarily as the reciprocal of the serum dilution corresponding to an optical density of 0.3. The minimum detectable titer was 100.

Mice and immunizations

C57BL/6 mice were purchased from Harlan Olac (Bicester, U.K.) and were used between 6 and 8 wk of age. IL-12 p40−/− (20) and IFN-γ−/− (21) gene knockout mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred at the University of Hull (Hull, U.K.) under containment conditions. Gene knockout mice were used between 6–12 wk of age. For i.n. immunization, groups of mice (n = 5) were lightly anesthetized with gaseous halothane, and 30 μl of Ag in PBS applied to the nasal nares. Mice were i.n. immunized on day 0 and 14 and killed on day 21–23. For s.c. immunization, groups of mice (n = 3) were injected s.c. on the left side of the abdomen with 150 μl of Ag mixture in PBS. As per i.n. immunization, mice were s.c. immunized on day 0 and 14 and killed on day 21–23. For oral immunizations, Ags were diluted in 0.1 M sodium bicarbonate and administered in a 200 μl volume directly into the stomachs of anesthetized mice (n = 4) via a gavage needle. Mice were orally immunized on day 0, 9, and 18 and killed on day 25. A group of mice (n = 3) were orally coimmunized with 200 μl of an emulsion of CFA containing 20 μg of OVA257–264 7–14 days before killing served as a positive control group for the induction of OVA-specific CTL in all experiments. All mice were bled from the tail vein before being killed.
adjuvants for CTL, whole OVA, or OVA257–264 were used as model bystander Ags in immunization experiments. Standard 51Cr release assays were used to detect CTL activity from splenocytes and cells from the sCLN. In preliminary single dose i.n. immunization experiments, inconsistent OVA-specific CTL responses were elicited using LT and CT as adjuvants. This was overcome by boosting mice 14 days after the initial immunization.

Mice i.n. immunized twice, 14 days apart, with 1 μg of LT or CT mixed with 100 μg of OVA or 40 μg of OVA257–264 mounted OVA-specific CTL responses which were consistently detected in the spleens and sCLNs of immunized animals (Fig. 1). Mice immunized with 1 μg of LT mixed with 2 μg of OVA257–264 (an equimolar amount of OVA257–264 epitope to that present in 100 μg of whole OVA) also mounted OVA-specific CTL responses, which were detected in spleens (data not shown).

CTA1-DD is a novel ADP-ribosylating molecule with affinity for B cells. CTA1-DD has been shown to possess mucosal adjuvanting activity following i.n. administration with bystander Ags (8). CTA1-DD adjuvanted weak OVA-specific CTL responses and only when coadministered with whole OVA (Fig. 1). Intranasal immunization of mice with 100 μg of OVA alone or 40 μg of OVA257–264 alone consistently failed to elicit a detectable CTL response in either the spleen (Fig. 1) or the sCLN (data not shown).

**LT adjuvants MHC class I restricted CD8+ CTL responses**

To identify the T cell population with cytotoxic activity, either CD8+ or CD4+ T cells were depleted from restimulated spleen cell populations prepared from mice i.n. immunized with 1 μg of LT mixed with OVA or OVA257–264. Depletion of CD8+ T cells, but not CD4+ T cells, immediately before coculturing them with chromium-labeled targets abrogated all lytic activity (data not shown).

**LTR72 and LTK63 but not rLT-B adjuvant OVA-specific CTL responses after i.n. immunization**

The adjuvant properties attributed to LT are reportedly enhanced by the ADP-ribosyltransferase activity associated with the A subunit (4). The role of this enzymatic activity and LT-A itself in promoting CD8+ CTL responses to OVA or OVA257–264 was assessed in immunization studies using attenuated LT derivatives with reduced (LTR72) or absent (LTK63 and rLT-B) enzymatic activity. Initial experiments using 1 μg of mutant toxin as adjuvant in i.n. immunization experiments failed to consistently elicit OVA-specific CTL responses. However, i.n. immunization of mice with 10 μg of LTK63 or 10 μg of LTR72 mixed with 100 μg of OVA or 40 μg of OVA257–264 elicited OVA-specific CTL responses which were reproducibly detected by 51Cr release assay (Fig. 2). In contrast, i.n. coimmunization of mice with 10 μg of LT-R mixed with 100 μg of OVA or 40 μg of OVA257–264 failed to elicit significant CTL responses (Fig. 2).

OVA257–264 peptide-specific IFN-γ ELISpot assays were performed in parallel with 51Cr-release assays using splenocytes from selected groups of i.n. immunized mice. The results (Fig. 2D) demonstrated that i.n. coadministration of OVA or OVA257–264 with LT or LTK63, but not rLT-B, elicited OVA257–264-specific T cells which produced IFN-γ upon secondary restimulation in vitro.

**LT, CT, and LTR72, but not LTK63 or LT-B, adjuvant OVA-specific CTL responses after oral immunization**

The capacity of LT derivatives to adjuvant OVA-specific CTL responses by the oral route of immunization was explored. LT, and to a lesser extent a CT control, adjuvanted splenic CTL responses after oral immunization of 10 μg of toxin with 5 mg of OVA (Fig. 3). The OVA-specific CTL responses elicited using 10 μg of LTR72 as adjuvant were comparable to that elicited by 10 μg of wild-type LT (Fig. 3). In contrast, oral administration of LTK63 or rLT-B with 5 mg of OVA failed to elicit detectable CTL responses (Fig. 3). Oral immunization of mice with 5 mg of OVA alone failed to elicit a CTL response (Fig. 3).

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**FIGURE 1.** ADP-ribosylating molecules adjuvant OVA specific CTL responses after i.n. immunization. C57BL/6 mice (groups of 3) were i.n. immunized on day 0 and day 14 and killed on day 21 when pooled spleen cells and cells from the sCLNs were cocultured with irradiated E.G7-OVA cells for 6 days. Restimulated spleen cells were assessed against 51Cr-labeled EL4 (∆) and E.G7-OVA (●) cells. Immunization of 1 μg of LT with 100 μg of OVA or 40 μg of OVA257–264 elicited OVA-specific CTLs in spleens (A) and sCLNs (B). Splenic CTLs were also detected when 1 μg of CT (C) or 20 μg of CTA1-DD (D) was used to adjuvant whole OVA; however, only CT adjuvanted OVA257–264. Immunization of mice with OVA alone or OVA257–264 alone failed to elicit detectable OVA-specific CTLs (E). The results are representative of two to four separate experiments.
LT, CT, LTR72, LTK63, and CTA1-DD, but not rLT-B, adjuvant OVA-specific CTL responses after s.c. immunization.

Mice immunized s.c. with 100 μg of OVA mixed with 1 μg of the ADP-ribosylating molecules LT or CT, or 20 μg of CTA1-DD, all mounted OVA-specific CTL responses that were detected in the spleen (Fig. 4). Similarly, mice immunized s.c. with 100 μg of OVA mixed with 1 μg of the attenuated toxins LTR72 or LTK63 also mounted OVA-specific CTL responses. Conversely, immunization of mice with 1 μg of rLT-B mixed with 100 μg of OVA, or 100 μg of OVA alone, failed to elicit a CTL response (Fig. 4).

The OVA-specific CD8+ CTL responses elicited using LT as adjuvant are independent of CD4+ T cell help.

CD4+ T cell help is a common but not universal requirement for CTL responses (24). The role of CD4+ T cells in the induction of OVA-specific CD8+ CTL responses using LT as an adjuvant was assessed by depleting mice of CD4+ T cells with the mAb GK1.5. Mice were injected twice with 0.5 mg of GK1.5 or rat IgG (control group) before being i.n. immunized twice, 14 days apart, with 1 μg of LT mixed with 100 μg of OVA or 40 μg of OVA257–264. Mice were injected again with 0.5 mg of the GK1.5 mAb or rat IgG on...
LT mixed with OVA or OVA257–264 (Fig. 5). In contrast, the serum Ab response to OVA, which in rat IgG-treated mice exceeded a titer of 10^5, was completely abrogated in CD4-depleted mice (Fig. 5K). Mice administered GK1.5 or rat IgG and i.n. immunized with 100 μg of OVA alone or 40 μg of OVA257–264 alone failed to mount significant OVA-specific CTL responses (Fig. 5). As previously described (25), OVA-specific CTL responses resulting from s.c. immunization of mice with OVA257–264 emulsified in CFA were independent of CD4^+ T cell help (Fig. 5).

**OVA-specific CD8^+ CTL responses elicited using LT as mucosal adjuvant are independent of IL-12 and IFN-γ**

IL-12 and IFN-γ play central roles in the development of Th1-type immune responses and, potentially, in Th cell-independent CTL generation (26). The contribution of IL-12 and IFN-γ to the induction of OVA-specific CD8^+ CTL responses was assessed in mice deficient in the IL-12 p40 subunit and IFN-γ gene respectively. C57BL/6 mice (H-2^b), IL-12 p40^−/− mice (H-2^b) and IFN-γ^−/− mice (H-2^b) were immunized i.n. with 1 μg of LT mixed with 100 μg of OVA or 40 μg of OVA257–264. Additional groups of normal and gene knockout mice were immunized i.n. with 100 μg of OVA alone or 40 μg of OVA257–264 alone.

In multiple experiments, i.n. immunization of IL-12 p40^−/− and IFN-γ^−/− mice elicited OVA-specific CTL responses that were similar in magnitude, as shown by 51Cr release assay, to that detected in C57BL/6 control mice (Fig. 6). The induction of OVA-specific T cell responses in IL-12 p40^−/− mice was also assessed by IFN-γ and IL-4 ELISPOT. The IFN-γ ELISPOT results indicated that IL-12 p40^−/− mice, i.n. immunized with LT mixed with OVA or OVA257–264, had fewer numbers of OVA257–264 peptide-specific IFN-γ SFCs when compared with normal control mice. However, these differences were not statistically different (Fig. 7). In contrast, IL-12 p40^−/− mice immunized with OVA257–264 emulsified in CFA had significantly fewer peptide-specific splenic T cells producing IFN-γ when compared with similarly immunized normal control mice (p < 0.05) (Fig. 7).

The frequency of IL-4 positive SFCs obtained with unstimulated splenocytes from i.n. immunized IL-12 p40^−/− (80 ± 32 SFCs per 10^6 splenocytes), or IFN-γ^−/− mice (58 ± 46 SFC/10^6 splenocytes), was not significantly different to that found in i.n. immunized C57BL/6 mice (86 ± 34 SFC/10^6 splenocytes). The frequency of IL-4 SFCs did not increase when spleen cells from immunized IL-12 p40^−/− mice, IFN-γ^−/− mice, or normal C57BL/6 mice were restimulated with OVA257–264 (data not shown).

**Discussion**

CTLs represent an important immune effector mechanism for resistance and protection against many viral and some bacterial infectious agents. The induction of CTL responses to appropriate Ags from relevant pathogens is therefore an aim of many novel immunization approaches. The results of this study suggest that i.n. codelivery of peptides or protein Ags with attenuated LT mutants represents a viable CTL immunization strategy. In this report, we showed that LT mutants with reduced (LTR72) or undetectable (LTK63) levels of ADP-ribosyltransferase activity adjuvanted OVA-specific CTL responses after i.n. coimmunization with either whole OVA or OVA257–264. Both LTR72 and LTK63 also adjuvanted OVA-specific CTL responses after s.c. comminunization with OVA, but only LTR72 elicited CTL responses after oral coimmunization. LT-B was unable to adjuvant CTL activity following immunization by any route. Further, it was demonstrated that LT-mediated OVA-specific CTL responses were independent of

day 5, 10, and 15 postprimary immunization. The efficiency of CD4^+ T cell depletion was assessed on day 0, 10, and 21 by flow cytometric analysis (using a noncompeting anti-CD4 mAb) of lymphocytes recovered from the spleens and sCLNs of randomly selected mice. In two separate experiments, between 93 and 96% of CD4^+ T cells were depleted from both the spleen and sCLN at the time points studied.

Depletion of CD4^+ T cells did not prevent the induction of an OVA-specific CD8^+ CTL response in mice i.n. immunized with
OVA 257–264 emulsified in CFA s.c. All mice were killed on day 21, and were i.n. immunized on days 0 and day 14 with 1 \text{ alent amount of rat IgG before and during the immunization protocol. Mice}

The availability of recombinant derivatives of LT which are either devoid or reduced in enzymatic activity (LTK63 and LTR72), or which lack the enzymatic A domain (rLT-B) has facilitated a dissection of the properties of this toxin which endow it with adjuvant activity. The inability of rLT-B to adjuvant CTL responses to i.n., orally, or s.c. codelivered OVA suggests that the A subunit of LT endows the toxin with characteristics essential for promoting CTL responses. The ADP-ribosyltransferase activity associated with the A subunit in wild-type LT appears to enhance the adjuvant potential of the toxin, but is nevertheless nonessential for adjuvanticity because LTK63, which is devoid of ADP-ribosyltransferase activity, adjuvants mucosal and systemic Ab (5), and CTL responses to i.n. or s.c. coadministered OVA. The function of the mutated A subunit in LTK63 may be to enhance the half-life of the holotoxin in vivo and thereby increase the host exposure to the affects mediated by LTK63 binding its ubiquitously found glycosphingolipid receptors. These effects may include up-regulated expression of MHC class II and co-stimulatory molecules on B cells (27) or stimulating cellular synthesis of arachidonic acid metabolites (e.g., PGE$_2$) (28). Alternatively, the A subunit in LTK63 may possess other immunomodulating properties which, in addition to affects mediated by toxin ligation of receptor, are critical for adjuvancing CTL responses to bystander proteins or peptides. These immunomodulatory properties could potentially be mediated through binding of the A subunit to ADP-ribosylation factors (ARFs), an interaction that has been shown to occur outside the NAD binding cleft (29). While speculative, ARF binding to the A subunit may lead to alterations in vesicular membrane trafficking in both endocytic and exocytic pathways of host cells, because ARFs are recognized as being essential mediators of these events (30, 31).

The results of this study indicated that LTK63 was unable to adjuvant splenic CTL responses after oral immunization with OVA. It is unclear whether this represents a definitive inability to adjuvant CTL responses perorally, or whether a CTL response may be elicited by increasing the amount of LTK63 coadministered with the bystander Ag. In any case, the result suggests that toxins with either partial (e.g., LTR72) or wild-type levels (e.g., LT, CT) of ADP-ribosyltransferase activity are better oral adjuvants for CTL responses.

The novel finding that CTA1-DD and CT can adjuvant CTL responses after i.n. coadministration with whole OVA alone (A and B) or 100 \mu g of OVA alone (C and D) did not mount strong OVA-specific CTL responses. OVA-specific CTLs were detected in the spleens of mice immunized with 1 \mu g of LT mixed with 40 \mu g of OVA$_{257-264}$(E and F) or 100 \mu g of OVA (G and H), irrespective of whether they had been depleted of CD4$^+$ T cells or not. Mice s.c. immunized with 20 \mu g of OVA$_{257-264}$ emulsified in CFA mounted OVA-specific CTLs (I and J) as previously described (25). K, Depletion of CD4$^+$ T cells abrogates the serum Ab response to OVA after i.n. coadministration with LT. Anti-OVA Ab titers in sera of mice i.n. immunized with 1 \mu g of LT mixed with 100 \mu g of OVA or 100 \mu g of OVA alone were analyzed on day 21. The data depict the anti-OVA serum Ab titers of individual mice. Mice treated with rat IgG before and during immunization had high anti-OVA serum Ab titers (K). In contrast the serum anti-OVA Ig response was abrogated in mice depleted of CD4$^+$ T cells (K). The results shown were pooled from two separate experiments.
highlights the immunological potency of molecules with ADP-ribosyltransferase activity. CTA1-DD is a potent activator of B cells and strongly up-regulates costimulatory molecules, in particular B.7, on their surface (8). That the CTA1-DD adjuvant fails to prime CD4^+ T cells in B cell-deficient mice, whereas it is highly effective in wild-type mice (N. Lycke, unpublished observation), indicates that this targeted adjuvant appears to act via B cells to induce naive CD4^+ T cells. This mechanism of T cell induction may also apply to CTA1-DD in its effect as an adjuvant for OVA-specific MHC class I-restricted CTL responses.

The capacity of CT to augment CTL responses to OVA_257–264 after i.n. immunization, or whole OVA after oral coimmunization, concurs with previous studies of this toxin (15, 16). Interestingly, although numerous studies have described CT as a potent inducer of Th2-type immune responses (6, 32), this study, like others (15, 16, 33), showed CT to be a useful adjuvant for CTL responses; a response not normally associated with strong Th2-type immune responses.

CD4^+ T cell lymphocytes play a central role in the regulation of the immune response. The role of CD4^+ T cells in the induction and maintenance of CD8^+ CTL responses appears to depend on the system in which they are studied. CD8^+ CTL responses which result from cross priming, as occurs after DNA vaccination (34), or injection of cell associated Ags (25, 35), is clearly dependent on CD4^+ T cell help. Elegant studies indicate that this CD4^+ T cell help is mediated through CD154 signaling of CD40 on the host APC (35–37). In studies of CTL responses to certain viruses however, CD4^+ T cell help is not critical for the induction of CD8^+ CTL, but they may contribute to maintenance of memory CTL responses (38, 39). Similarly, induction of CD8^+ CTL responses by injection of class I binding peptides in strong adjuvants is not dependent on CD4^+ T cell help (25), but their long-term maintenance can require CD4^+ T cells (40). The results of this study showed that LT, like CFA, can elicit CTL responses to OVA or OVA_257–264 independently of CD4^+ T cell help. This result suggests that LT directly or indirectly activates host APCs (thereby obviating the need for this activation to occur via CD4^+ T cells), such that they are now competent to prime an OVA-specific CD8^+ T cell response. The identity of the APC which primes the CD8^+ T cell response after i.n immunization with CT and OVA_257–264 has been suggested in a previous study. Porgador et al. (41) showed that following intranasal coimmunization of OVA_257–264 with CT, only dendritic cells (DCs) from the nasal associated lymphoid tissue could present OVA_257–264 to OVA-specific T cells in vitro, suggesting that DCs can also present antigenic peptides from soluble OVA has not been determined, although previous studies have established that DCs can take up OVA via macropinocytosis in vitro and subsequently prime an Ag-specific CTL response following adoptive transfer in vivo (42).
expressing IFN-γ potentiate OVA-specific CTL responses independently of IFN-γ that the requirement for IL-12 in priming T cells which express have also been described (48, 50), and together these results imply an IL-12-independent mechanism through which CD8\(^+\) T cells. Furthermore, our IFN-γ ELISPOT results suggest the presence of an IL-12-independent mechanism through which CD8\(^+\) T cells expressing IFN-γ (but not IL-4) can be elicited (Fig. 7). IL-12-independent mechanisms of inducing Th1 CD4\(^+\) T cell responses have also been described (48, 50), and together these results imply that the requirement for IL-12 in priming T cells which express IFN-γ is dependent on the nature and composition of the Ag and the cytokine environment in which Ag is presented to naive CD8\(^+\) T cells.

Like IL-12, IFN-γ has also variously been described as essential (51) and alternatively, dispensable for the induction of CTL in vivo (52). Our data, using IFN-γ\(^−/−\) mice, suggests that LT (and CFA) potentiate OVA-specific CTL responses independently of IFN-γ, and also imply that the cytokotic function of these T cells is not impaired by their inability to make IFN-γ. IFN-γ\(^−/−\) mice with acute viral infections (53, 54) also mount normal CTL responses, suggesting IFN-γ may be unimportant or redundant for CD8\(^+\) CTL responses when Ag is presented in an environment rich in inflammatory cytokines and mediators. Interestingly, following immunization of IFN-γ\(^−/−\) mice, the number of OVA\(_{257-264}\) specific CD8\(^+\) T cells was not biased toward expression of a typical type II cytokine.

There exist many experimental strategies that can be used to elicit Ag-specific CTL responses in model systems. The attractiveness of using molecularly defined, nontoxic adjuvants based on LT lies in their capacity to elicit CTL responses by mucosal immunization. That mucosally elicited CTL mediates more effective resistance to a mucosal pathogen than systemically elicited CTL has already been demonstrated in one system (14). The authors are currently extending this area of research using nontoxic derivatives of LT.

**References**


