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Cholera Toxin Promotes the Induction of Regulatory T Cells Specific for Bystander Antigens by Modulating Dendritic Cell Activation[1]

Ed C. Lavelle, Edel McNeela, Michelle E. Armstrong, Olive Leavy, Sarah C. Higgins, and Kingston H. G. Mills[2]

It has previously been reported that cholera toxin (CT) is a potent mucosal adjuvant that enhances Th2 or mixed Th1/Th2 type responses to coadministered foreign Ag. Here we demonstrate that CT also promotes the generation of regulatory T (Tr) cells against bystander Ag. Parenteral immunization of mice with Ag in the presence of CT induced T cells that secreted high levels of IL-4 and IL-10 and lower levels of IL-5 and IFN-γ. Ag-specific CD4$^+$ T cell lines and clones generated from these mice had cytokine profiles characteristic of Th2 or type 1 Tr cells, and these T cells suppressed IFN-γ production by Th1 cells. Furthermore, adoptive transfer of bone marrow-derived dendritic cells (DC) incubated with Ag and CT induced T cells that secreted IL-4 and IL-10 and low concentrations of IL-5. It has previously been shown that IL-10 promotes the differentiation or expansion of type 1 Tr cells. Here we found that CT synergized with low doses of LPS to induce IL-10 production by immature DC. CT also enhanced the expression of CD80, CD86, and OX40 (CD134) on DC and induced the secretion of the chemokine, macrophage inflammatory protein-2 (MIP-2), but inhibited LPS-driven induction of CD40 and ICAM-I expression and production of the inflammatory cytokines/chemokines IL-12, TNF-α, MIP-1α, MIP-1β, and monocyte chemoattractant protein-1. Our findings suggest that CT induces maturation of DC, but, by inducing IL-10, inhibiting IL-12, and selectively affecting surface marker expression, suppresses the generation of Th1 cells and promotes the induction of T cells with regulatory activity. *The Journal of Immunology,* **2003, 171: 2384–2392.**

CD4$^+$ T cells that express CD25 or secrete high levels of immunosuppressive cytokines, termed regulatory T (Tr)[3] cells, are known to play a role in tolerance to self Ag and in the prevention of autoimmune diseases (1). As well as a role in maintaining immune homeostasis, evidence is now emerging that Tr can be induced by infectious pathogens, either as an evasion strategy to subvert protective Th1 responses or as a protective mechanism of the host to limit pathogen-induced immunopathology (2). T cells that secrete IL-10 and/or TGF-β, but no IL-4, termed type 1 Tr (Tr1) cells or Th3 cells, have been generated against foreign Ag (3–6). Tr cells suppress immune responses mediated by CD25$^-$ T cells or Ag-specific Th1 or Th2 cells by direct cell-to-cell contact or through the secretion of IL-10 or TGF-β (1–7). Although the induction of Tr cells may not be desirable in vaccination against infectious diseases, where Th1 or Th2 responses are known to mediate protection, Tr cells have considerable potential in the treatment of immune-mediated diseases. Tr cells have been shown to prevent autoimmune diseases mediated by Th1 cells (3, 7). However, these cells are difficult to propagate in vitro, and their therapeutic potential is dependent on the identification of strategies for their induction and expansion in vivo.

Th1 or Th2 cells specific for foreign Ag can be induced using adjuvants based on pathogen-derived molecules that promote regulatory cytokine production and dendritic cell (DC) maturation through binding to pathogen recognition receptors, including Toll-like receptors (TLRs) (8). Certain TLR ligands, including CpG motif-containing oligonucleotides (CpG-ODN), LPS and poly(I: C), stimulate IL-12 production by DC and macrophages and promote the induction of Th1 cells (9, 10). In contrast, filamentous hemagglutinin from *Bordetella pertussis* enhances IL-10 production from macrophages and DC and promotes the induction of Tr1 cells (4). Furthermore, products of helminth parasites and cholera toxin (CT) have been shown to activate DC, which promote the differentiation of Th2 cells (11, 12).

CT is an enterotoxin produced by *Vibrio cholerae* consisting of a toxic A subunit noncovalently linked to a pentamer of cell-binding B subunits. The B subunits bind to ganglioside GM1 receptors, facilitating intracellular entry of the A subunit. The ADP-ribosylating A subunit activates Gsα, a GTP-binding protein, which stimulates adenylyl cyclase, leading to enhanced intracellular cAMP, thereby inducing a multiplicity of biological effects (13). CT can also ADP-ribosylate other G proteins, and thus affect cellular processes in addition to those regulated by cAMP. The principal intracellular target of cAMP is protein kinase A (PKA), but recent data have demonstrated that cAMP can also activate signaling pathways independently of PKA (14).

CT is a powerful adjuvant, and most studies in rodent models have concluded that coimmunization with CT predominantly enhances Th2 responses to coadministered Ag. These responses are

characterized by CD4$^+$ T cell production of IL-4, IL-5, IL-6, and IL-10 and IgG1, IgA, and IgE Abs (15), but elevated IFN-γ and IgG2a Abs have also been reported (16). Anti-inflammatory effects of CT and the closely related toxin, heat-labile enterotoxin from *Escherichia coli* (LT), have also been documented, in particular down-regulation of IL-12 p70 and TNF-α production by innate immune cells in response to LPS (17, 18). Furthermore, CT and LT can suppress IFN-γ production (18) and autoimmune diseases mediated by Th1 cells (19). In the present study we examined the possibility that the anti-inflammatory effects of CT may extend to the induction of Tr cells specific for bystander Ag through modulation of DC function. Our findings suggest that CT can promote the induction of regulatory T cells to coadministered Ag by enhancing the production of IL-10 and inhibiting the activation of Th1-inducing DC.

Materials and Methods

Materials

CT was purchased from Sigma-Genosys (Poole, U.K.). The protein was highly contaminated with LPS (>1000 endotoxin units/mg) and was purified on endotoxin removal columns (Detoxi-Gel endotoxin removing gel; Pierce, Rockford, IL). Following this step, endotoxin was undetectable in the preparation using the chromogenic *Limulus* amebocyte lysate assay (BioWhittaker, Walkersville, MD). Depyrogenated keyhole limpet hemocyanin (KLH) was purchased from Calbiochem (La Jolla, CA). Phosphorothioate-stabilized CpG-ODN (5′-GCTAGACGTTAGCGT-3′) was synthesized by Sigma-Genosys (Cambridge, U.K.). *E. coli* LPS (serotype 127: B8) and poly(I:C) were purchased from Sigma-Genosys (Poole, U.K.). All solutions were prepared in pyrogen-free water (Baxter, Maychem Sales, Dublin, Ireland) or Dulbecco's PBS (Sigma-Genosys). The endotoxin contents of all modulators and inhibitors used were determined using the chromogenic *Limulus* amebocyte lysate assay.

Animals and immunization

Female BALB/c, C3H/HeN, and C3H/HeJ mice were obtained from Harlan Olac (Bicester, U.K.) and were used at 8–10 wk of age. Animals were maintained according to the regulations of the European Union and the Irish Department of Health. Mice were immunized s.c. in the footpad with KLH (10 μg) or KLH (10 μg) and CT (1.0 μg) and were boosted 7 days later with KLH (20 μg). Mice immunized with PBS served as controls.

Isolation and culture of bone marrow-derived DC

Bone marrow-derived immature DC were prepared by culturing bone marrow cells obtained from the femurs and tibia of mice in RPMI 1640 medium and 10% FCS supplemented with 10% supernatant from a GM-CSF-expressing cell line (J558-GM-CSF). The cells were cultured for 3 days, pelleted by centrifugation (200 × g, 5 min), and recultured with fresh medium and 10% GM-CSF cell supernatant on day 3. On day 7 of culture, cells were collected, washed, and used for assays.

Generation of Ag-specific T cell lines and clones

Spleen or popliteal lymph node (LN) cells (2 × 10^6/ml) from immunized mice were cultured with KLH (50 μg/ml). After one round of Ag stimulation, T cell lines were cloned by limiting dilution as previously described (4). T cell lines and clones were maintained by culture with Ag (KLH, 50 μg/ml) and splenic APC for 4–5 days, followed by 5–7 days of culture with irradiated feeder cells and IL-2. T cells were tested for Ag specificity and cytokine production at the end of the starve cycle.

Adoptive transfer of modulated Ag-pulsed DC

DC (10^6/ml) were incubated for 2 h with KLH (10 μg/ml), KLH and CT (1 μg/ml), or medium alone. Cells were extensively washed, and 10^5 cells were injected into the footpads of mice. After 7 days, spleens and popliteal LN were removed to test for KLH-specific cytokine production, and serum was recovered to assess KLH-specific Ab production.

Ag-specific cytokine production

Spleen (2 × 10^6 cells/ml) or popliteal LN (1 × 10^6 cells/ml) cells from immunized mice or T cell lines or clones and APC (irradiated spleen cells, 2 × 10^6/ml) were cultured in complete RPMI medium at 37°C and 5% CO$_2$ with KLH (2–50 μg/ml) or PMA (Sigma-Genosys; 20 ng/ml) and anti-

mouse CD3 (BD PharMingen, San Diego, CA; 1 μg/ml). Supernatants were collected after 72 h, and concentrations of IL-4, IL-5, IL-10, IL-13, and IFN-γ were determined by immunoassay using Ab pairs specific for IL-4, IL-5, and IFN-γ (all from BD PharMingen), and IL-10 and IL-13 (Duo-Set; R&D Systems, Minneapolis, MN). The effect of Tr1 cells (generated from mice immunized with KLH in the presence of CT) on cytokine production by Th1 cells (generated from mice immunized with KLH in the presence of CpG-ODN) was assessed in coculture experiments. KLH-specific Tr1 lines (1 × 10^4 to 1 × 10^6/ml) and KLH-specific Th1 lines (1 × 10^5/ml) were cultured alone or together with APC and Ag (KLH, 50 μg/ml). Supernatants were removed after 3 days and were tested for cytokine production.

Ab assays

Titers of KLH-specific IgG and IgG subclasses in serum were determined by ELISA as previously described (18).

Effect of CT on DC maturation and cytokine production

Bone marrow-derived DC (1 × 10^6/ml) were cultured at 37°C for 30 min to 24 h with CT (10–10,000 pg/ml); LPS (100 pg/ml to 1 μg/ml); CpG-ODN (1 ng/ml to 10 μg/ml); poly(I:C) (1 ng/ml to 10 μg/ml); CT in the presence of LPS, CpG-ODN, or poly(I:C); or medium alone. In certain experiments DC were incubated with modulators in the presence or the absence of a neutralizing anti-IL-10 mAb (10 μg/ml). At the end of the incubation period, supernatants were removed, and cytokine and chemokine concentrations were determined by immunoassays. Alternatively cells were recovered after 24 h, washed, and used for immunofluorescence analysis. IL-12p40, IL-12p70, IL-10, TNF-α, CCL2 (monocyte chemoattractant protein-1 (MCP-1)), CCL3 (macrophage inflammatory protein-1α (MIP-1α)), CCL4 (MIP-1β), and MIP-2 concentrations were measured by immunoassays using pairs of Abs purchased from BD PharMingen or R&D Systems (Abingdon, U.K.). The expression of DC surface markers was assessed using anti-mouse CD40 (rat IgG$_{2a}$, clone 3/23), I-A^d (mouse IgG2b, clone AMS-32.1), CD80 (hamster IgG2, clone 16-10A1), CD86 (rat IgG2a, clone GL1), CD11c (hamster IgG1, clone HL3), and CD134 (OX40; rat IgG1, clone OX86; Serotec, Oxford, U.K.). Cells incubated with appropriately labeled, isotype-matched Abs with irrelevant specificity acted as controls. Unless otherwise stated, all Abs were purchased from BD PharMingen. After incubation for 30 min at 4°C (followed by washing and incubation with streptavidin-PerCP for 20 min in the case of biotin-labeled primary Abs), cells were washed, and immunofluorescence analysis was performed on a FACScan (BD Bioscience, San Jose, CA) using CellQuest software. Thirty thousand cells were analyzed per sample.

Detection of cytokine mRNA in CT activated DC by RT-PCR

DC were cultured at 3 × 10^6 cells/ml in 6-well plates (Nunc, Naperville, IL) for 12 h and then stimulated with CT (1 μg/ml), LPS (1 μg/ml), CT and LPS, or medium alone. DC were harvested after 30 min to 12 h, supernatant was discarded, and 1 ml of Tri-Reagent (Sigma-Genosys) was added. Total RNA was extracted from cells according to the manufacturer's instructions. First-strand cDNA was synthesized from 2 μg of total RNA and was used as a template for PCR amplification with primers (5′-3′ forward, reverse) specific for IL-10 (CTGGACAACATACTGCTAACCGAC and TTCATTCATGGCCTTGTAGACACC), IL-12p40 (TCGCAGCAAAGC AAGATGTG and GAGCAGCAGATGTGAGTGGC), TNF-α (TGAAC TTCGGGGTGATCGGTC and AGCCTTGTCCCTTGAAGAGAAC), MIP-2 (TGGGTGGGATGTAGCTAGTTCC and AGTTTGCCTTGACC CTGAAGCC), or β-actin (GGACTCCTATGTGGGTGACGAGG and TCTTTGCCAATAGTGATGACTTGGC), which was used as a housekeeping gene. Oligonucleotide primers (50 pmol of each) and cDNA template were incubated in PCR buffer (Promega, Southampton, U.K.), 1.5 mM MgCl$_2$, 0.2 mM of each dNTP (Bioline, London, U.K.), and 2.5 U of *Taq* polymerase (Promega). The reaction mixture was incubated in a Mastercycler gradient thermal cycler (Eppendorf, Cambridge, U.K.) at 95°C for 5 min, then 30–35 cycles at 94°C for 1 min, 65°C for 1 min, and 72°C for 2 min were performed, followed by a final extension at 72°C for 10 min. Amplified PCR products were separated by electrophoresis on 1.2% agarose gels, and all PCR products were of the expected size (IL-10, 300 bp; IL-12 p40, 623 bp; TNF-α, 295 bp; MIP-2, 466 bp; β-actin, 605 bp). All primer pairs used spanned at least one intron to ensure amplification of mRNA product of a different size from contaminating DNA products. Furthermore, to demonstrate the absence of genomic DNA contamination of RNA samples during RT reactions, RNAs were processed in parallel without Moloney murine leukemia virus reverse transcriptase enzyme.

FIGURE 1. CT induces Tr1/Th2 responses to a coadministered Ag. Female BALB/c mice ($n = 5$) were immunized s.c. in the footpad with PBS, KLH (10 μg), or KLH (5 μg) and CT (1 μg). After 7 days, mice were boosted with PBS or KLH (10 μg). Popliteal LN cells, isolated 7 days later, were stimulated with KLH (2–50 μg/ml) or medium alone. Supernatants were removed after 3 days and were tested for IL-4, IL-5, IFN-γ, and IL-10 by immunoassay. Results represent the mean ($\pm$SD) of five mice per group and are representative of more than five experiments. *, $p < 0.05$; **, $p < 0.01$ (KLH vs KLH plus CT).
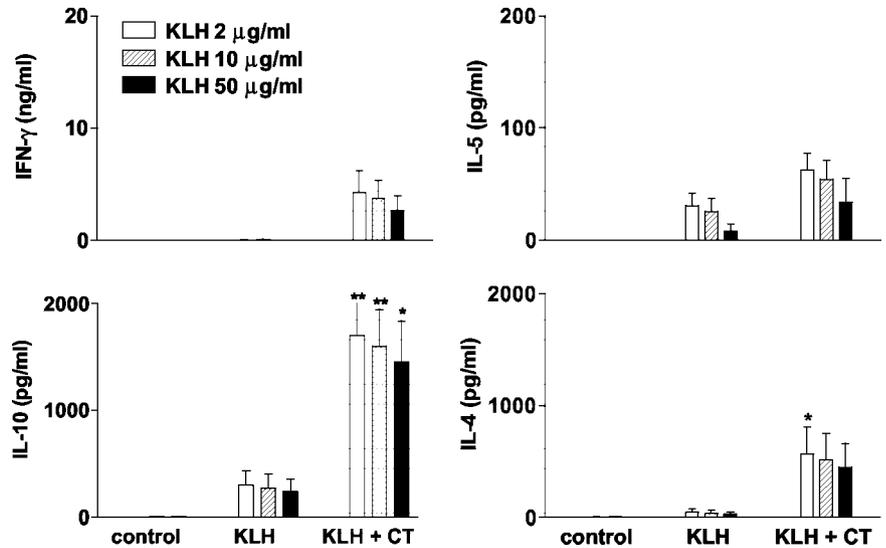
Statistics

Cytokine and chemokine levels 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. In cases when SDs were significantly different between groups, a nonparametric test (Kruskal-Wallis test with Dunn's multiple comparison post-test) was used to assess significance.

Results

CT promotes the induction of Tr1 and Th2 cells to bystander Ag in vivo

To examine the role of CT as a modulator, rather than a mucosal adjuvant, and in an attempt to compare direct immunization with transfer of modulated DC, we examined the ability of CT to enhance T cell responses to KLH administered by a parenteral route. Mice were immunized s.c. in the footpad with KLH alone or KLH

and CT, and immune responses were examined 7 days after a booster immunization with KLH. Spleen or LN cells from control mice immunized with PBS did not secrete any cytokines in response to KLH stimulation in vitro. Low levels of IL-4, IL-5, and IL-10 were detected in supernatants of Ag-stimulated cells from mice immunized with KLH alone. Compared with KLH alone, immunization with KLH in the presence of CT induced significantly higher levels of IL-4 and IL-10 by draining LN (Fig. 1) and spleen cells (not shown) and also generated a nonsignificant increase in IL-5 and IFN-γ production. Consistent with the predominant type 2 responses, the ratio of KLH-specific IgG1:IgG2a in the serum was 4.8:1.

To confirm that the cytokines detected were secreted by T cells and in an attempt to dissociate Th2 from Tr1 cells as a source of the Ag-specific IL-10, T cell lines were established from the
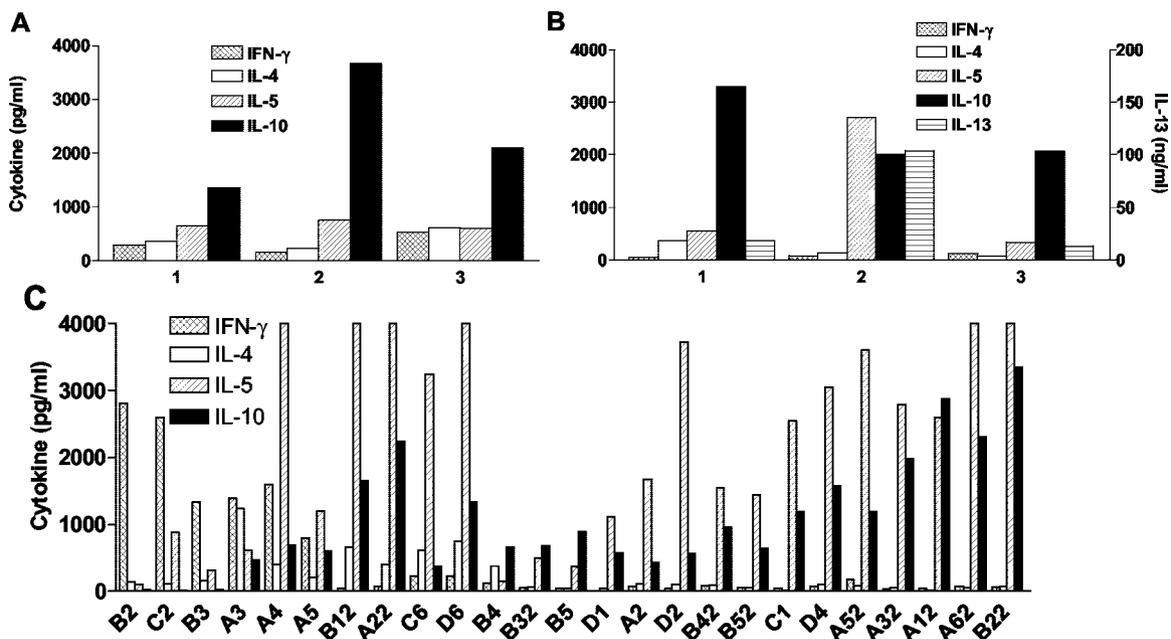
FIGURE 2. Ag-specific Tr1 and Th2 clones established from mice immunized with KLH and CT. T cell lines generated from the spleens of mice immunized with KLH and CT were tested for cytokine production after two (*A*) or three (*B*) rounds of Ag stimulation. *C*, T cell clones were established from these T cell lines by limiting dilution. T cell lines or clones were stimulated with KLH (50 μg/ml) in the presence of autologous APC, and cytokine concentrations were measured in the supernatants after 3 days.

FIGURE 3. T cells induced by immunization with Ag and CT suppress IFN-γ production by Th1 cells. KLH-specific T cell lines (1×10^4 to 1×10^6/ml) generated from mice immunized with KLH and CT (*A* and *B* correspond to T cell lines 1 and 2 described in Fig. 2*B*) and a KLH-specific Th1 cell line (1×10^5/ml) were cultured alone or together in the presence of APC (2×10^6/ml) and KLH ($50~\mu$g/ml). Supernatants were r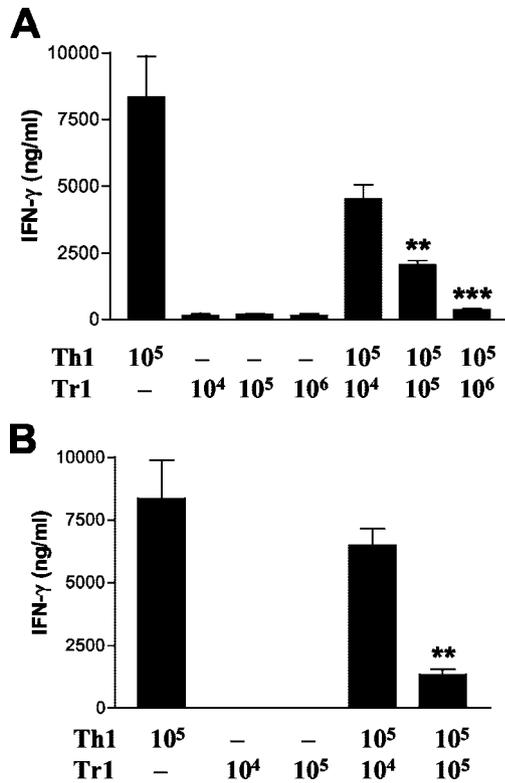emoved after 3 days, and IFN-γ concentrations were determined by immunoassay. Results are the mean ($\pm$SD) for triplicate cultures. **, $p < 0.01$; ***, $p < 0.001$ (Th1 vs Th1 plus Tr1).

spleens of immunized mice. KLH-specific CD4$^+$ T cell lines were successfully established from the spleens of mice immunized with KLH and CT and boosted with KLH. After two rounds of Ag stimulation, these T cell lines secreted high levels of IL-10, lower levels of IL-4 and IL-5, and very low levels of IFN-γ (Fig. 2*A*). After three rounds of Ag stimulation, the cytokine profile was more polarized to a Tr1-type profile, with IL-10 and IL-5, but low

or undetectable IL-4 and IFN-γ. The T cell lines also secreted IL-13 (Fig. 2*B*). T cell lines were cloned by limiting dilution after a single round of Ag stimulation in vitro, and a total of 25 CD4$^+$ T cell clones were successfully propagated (Fig. 2*C*). Fourteen of the T cell clones secreted IL-10 (642–3333 pg/ml) and IL-5 (368–4000 pg/ml), but low or undetectable IFN-γ or IL-4 (<200 pg/ml; even in the presence of anti-IL-4 receptor Ab), a cytokine profile characteristic of Tr1 cells. Five T cell clones were Th2 type (IL-4, 375–748 pg/ml; IL-5, 148-4000; IL-10, 375-2237 pg/ml), and the remaining clones had a mixed or Th0 profile, secreting IFN-γ and IL-5 with or without IL-4 and IL-10. The latter T cell clones are being recloned to confirm that they are monoclonal. Each of the T cell clones examined also secreted IL-13 (3.0–53.0 ng/ml).

Tr1-type cells generated in the presence of CT suppress IFN-γ production by Th1 cells

To examine the possible immunosuppressive function of the T cells generated by immunization with Ag in the presence of CT, we tested the ability of the T cell lines to exert bystander suppression of Th1 cells. Two Tr1/Th2 cell lines generated from mice immunized with KLH in the presence of CT were cocultured with a KLH-specific Th1-type T cell line generated from mice immunized with KLH in the presence of CpG-ODN. The T cell lines from the mice immunized with KLH in the presence of CT secreted high levels of IL-10 (Fig. 3*A*: 1×10^4/ml, 99 $\pm$ 15 pg/ml; 1×10^5/ml, 2,197 $\pm$ 84 pg/ml; 1×10^6/ml, 12,470 $\pm$ 0 pg/ml; Fig. 3*B*: 1×10^4/ml, 48 $\pm$ 7 pg/ml; 1×10^5/ml, 779 $\pm$ 127 pg/ml) and low or undetectable IFN-γ in response to Ag and autologous APC (Fig. 3). The Th1 cell line secreted high levels of IFN-γ (Fig. 3), but undetectable IL-4, IL-5, or IL-10. Addition of increasing numbers of Tr1/Th2 cell lines resulted in dose-dependent inhibition of IFN-γ production by the Th1 cell lines. Inhibition was observed at a ratio of Tr1/Th2:Th1 of 1:10 and was highly significant ($p < 0.01$) at ratios of 1:1 (Fig. 3). These findings demonstrate that T cells generated by immunization with Ags in the presence of CT have suppressor activity against Th1 cells in vitro.

Role of DC in directing T cell responses with CT

Since DC are considered to be the critical APC for activating naive T cells in vivo, we examined the role of DC in the selective enhancement of T cell subtypes by CT. Immature bone marrow-derived DC were incubated for 2 h with Ag (KLH; 10 μg/ml) alone or in the presence of CT (1 μg/ml). Cells were washed and

FIGURE 4. Adoptive transfer of CT- and Ag-treated DC induces a Tr1/Th2 response. BALB/c mice ($n = 5$) were immunized s.c. in the footpad with DC that were incubated with medium only, KLH (10 μg/ml), or KLH (10 μg/ml) and CT (1 μg/ml) for 2 h. Mice were injected with 1×10^5 cells; after 7 days spleens were removed, and cells were stimulated with KLH (2–50 μg/ml) or medium alone. Supernatants were removed after 3 days and tested for IL-4, IL-5, IFN-γ, and IL-10 by immunoassay. Results represent the means ($\pm$SD) of five mice per group and are representative of two experiments. *, $p < 0.05$; ***, $p < 0.001$ (KLH vs KLH plus CT).
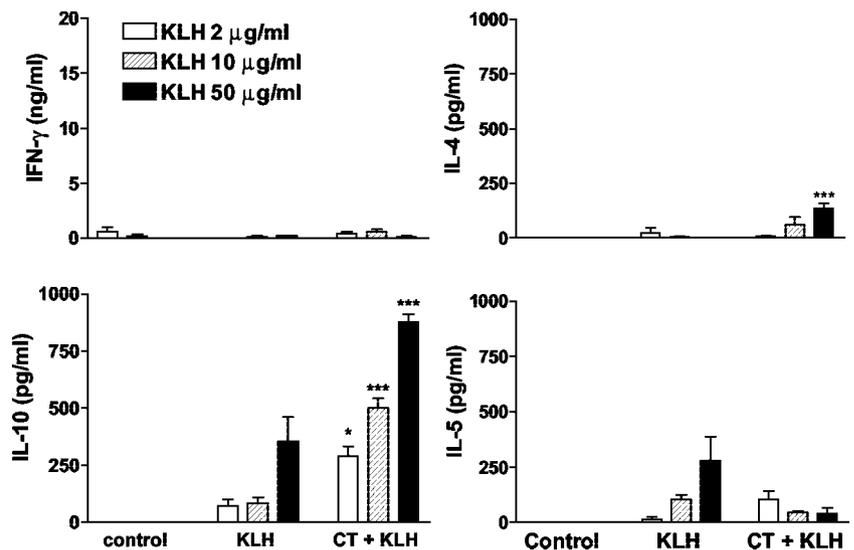
adoptively transferred to naive mice by s.c. injection into the foot-pad. Spleens were removed after 7 days, and Ag-specific cytokine production was determined. Spleen cells from mice injected with DC pulsed with KLH in the presence of CT secreted IL-10 and low levels of IL-4 and IL-5, but almost undetectable IFN-γ (Fig. 4). Ag-specific IL-10 and IL-4 production was significantly enhanced in spleen cells from mice injected with DC that were modulated with CT compared with DC pulsed with KLH alone. In contrast, KLH-specific responses could not be detected after transfer of un-treated DC. Transfer of KLH-pulsed CT-modulated DC also generated low levels of KLH-specific Ab production in the serum, which was enhanced following booster administration of soluble Ag or Ag-pulsed DC, and was predominantly IgG1 (data not shown).

CT synergizes with LPS in stimulating IL-10 production from DC

Since IL-10 has been shown to play a role in promoting the differentiation or expansion of Tr1 cells and has also been implicated in enhancing Th2 and inhibiting Th1 responses, we examined the possibility that CT could activate IL-10 production from DC. We found that CT alone failed to stimulate IL-10, but acted synergistically with LPS in inducing IL-10 production (Fig. 5). Incubation of DC with CT at concentrations of up to 10 μg/ml did not induce the production of IL-10. In contrast, coincubation of CT with LPS induced the rapid production of IL-10 at levels significantly higher than those induced with LPS alone. This effect was clearly synergistic, since the amount of IL-10 produced was significantly higher than the total of the amounts of the cytokine induced by LPS and the toxin individually. The synergy was apparent at the mRNA level from 30 min to 2 h after treatment (Fig. 5*B*) and at the protein level 2–12 h after stimulation of the DC with CT (Fig. 5*A*) and was more pronounced with lower doses of LPS. LPS induced dose-dependent production of IL-10 from DC in the concentration range 10–1000 ng/ml, which was significantly augmented by CT (Fig. 5*C*). IL-10 production with 1 ng/ml of LPS was close to the detection limit of the assay; however, significant levels of IL-10 were observed after addition of CT (Fig. 5*C*).

CT inhibits proinflammatory cytokine/chemokine production, but stimulates MIP-2 production

Stimulation of DC with LPS elicited production of the inflammatory cytokines, IL-12 p70 and TNF-α, and chemokines, MIP-1α, MIP-1β, MCP-1, and MIP-2 and also induced the anti-inflammatory cytokine, IL-10 (Fig. 5). In contrast, CT did not induce IL-12 p70, TNF-α, MIP-1α, MIP-1β, or MCP-1, but did induce the secretion of MIP-2 and, as reported above, IL-10 when LPS was also present (Fig. 6). CT-induced MIP-2 mRNA and protein was delayed until 12 h, compared with 2–4 h for LPS (data not shown).

Since CT enhances LPS-induced IL-10 from DC and since IL-10 is known to suppress proinflammatory cytokine production (20), we examined the influence of CT on LPS-induced inflammatory cytokines and chemokines. CT strongly inhibited LPS-induced IL-12 p70 production by DC, and this was observed when DC were incubated with CT before or at the same as the addition of LPS (Fig. 6 and data not shown). CT also significantly inhibited LPS-driven IL-12 p40, TNF-α, MIP-1α, MIP-1β, and MCP-1, but did not inhibit the production of MIP-2 (Fig. 6) or IL-6 (CT synergized with low doses of LPS to induce IL-6; data not shown).

We also examined the role of IL-10 in the suppressive effects of CT on inflammatory cytokine/chemokine production. Although below the detection limits of our ELISA, autocrine IL-10 appeared to exert a regulatory effect on DC. Measurable quantities of MIP-1α, MIP-1β, MIP-2, and TNF-α were detected in supernatants of

unstimulated cells incubated in the presence of anti-IL-10 (Fig. 6). Anti-IL-10 also significantly augmented LPS-induced IL-12 p70, TNF-α, and MIP-1α. However, the inhibitory effects of CT on LPS-driven IL-12 p40, TNF-α, MIP-1α, MIP-1β, and MCP-1 were only partially reversed by the addition of a neutralizing anti-IL-10 Ab (Fig. 6). Anti-IL-10 had no effect on CT inhibition of LPS-induced production of IL-12 p70, suggesting that the suppressive effect of CT was independent of IL-10.

As in the case of LPS, the TLR9 and TLR3 ligands, CpG-ODN and poly(I:C), elicited cytokine and chemokine production by DC. Coincubation with CT significantly inhibited CpG- and poly(I:C)-induced IL-12 p70, TNF-α, MIP-1α (Fig. 7), MIP-1β, and MCP-1, but did not inhibit the production of IL-6, IL-10, or MIP-2 (data not shown). Inhibition of inflammatory cytokine/chemokine production was observed over a range of concentrations of LPS and CpG-ODN (Fig. 7). Furthermore, the suppressive effects were observed with a dose as low as 1 ng/ml of CT (data not shown).

The inhibitory effect of CT on proinflammatory cytokine production was also demonstrated at the transcriptional level. Incubation of DC with CT alone failed to induce transcription of
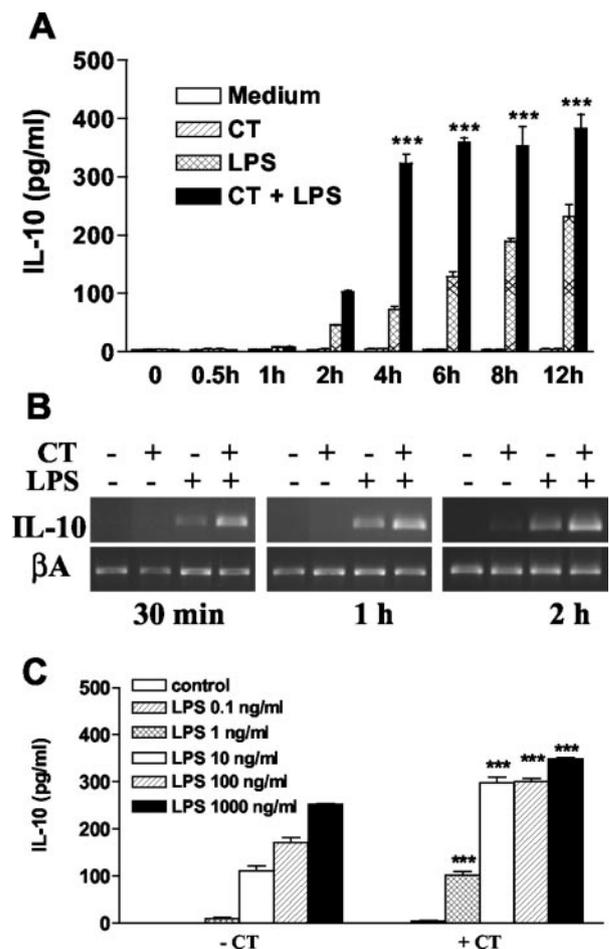
FIGURE 5. CT synergizes with LPS to enhance IL-10 production by DC. DC were incubated for 0.5–12 h with CT (1 μg/ml), LPS (10 ng/ml), or CT and LPS. IL-10 protein concentrations were determined in supernatants by immunoassay (*A*), and IL-10 mRNA was determined by RT-PCR (*B*). *C*, DC were incubated for 6 h with increasing doses of LPS (0.1–1000 ng/ml) alone or in the presence of CT (1 μg/ml), and supernatants were analyzed for IL-10 by immunoassay. RT-PCR results represent PCR products from three replicates for IL-10 mRNA compared with β-actin (βA). The results of cytokine protein assays are the mean ($\pm$SD) of triplicate assays and are representative of four experiments. ***, $p < 0.001$ (CT plus LPS vs LPS).

FIGURE 6. CT inhibits LPS-induced inflammatory cytokines and chemokines, but stimulates MIP-2 production. DC (1×10^6/ml) were incubated for 24 h with CT (1 μg/ml), LPS (1 μg/ml), CT and LPS, or medium alone in the presence or the absence of an anti-IL-10 Ab (10 μg/ml). Supernatants were assayed for cytokines by immunoassays. Results are representative of five experiments. $++$, $p < 0.01$; $+++$, $p < 0.001$ (LPS vs CT plus LPS). $***$, $p < 0.001$ (with vs without anti-IL-10). $\#\#\#$, $p < 0.001$ (CT vs medium control).
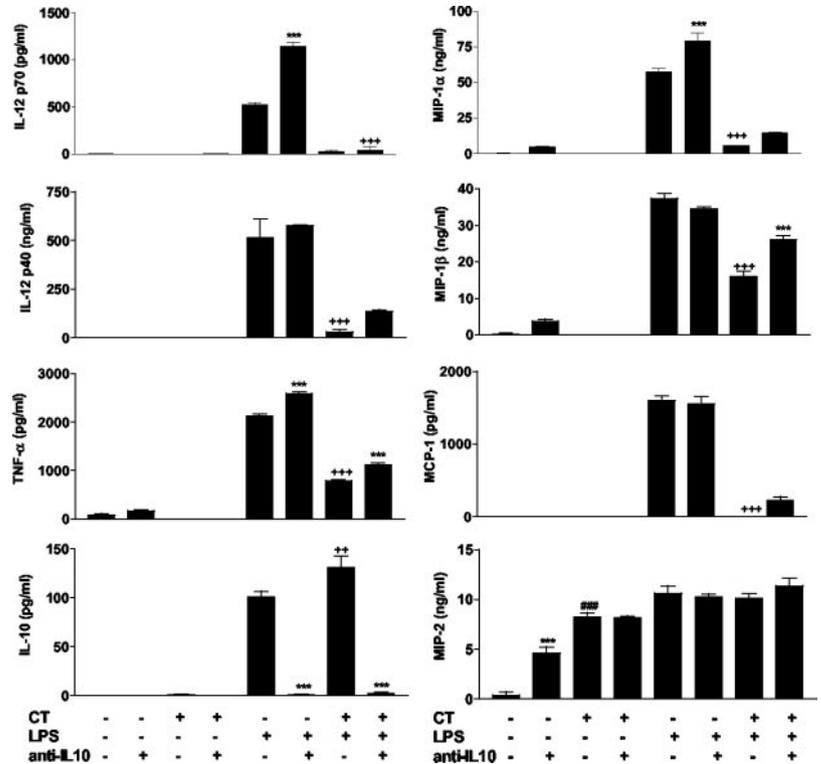
TNF-α or IL-12 p40, and indeed, constitutive levels of mRNA were reduced (Fig. 8). In contrast, LPS (10 ng/ml) stimulated the transcription of TNF-α mRNA within 1 h and that of IL-12 p40 mRNA within 4 h. Incubation of DC with CT inhibited LPS-induced transcription of TNF-α and IL-12 p40. The inhibitory effects were more pronounced at the later time points examined, especially for IL-12 p40 (Fig. 8).

Effect of CT on DC maturation

Having demonstrated that CT-treated Ag-pulsed DC can prime potent Ag-specific T cell and Ab responses following adoptive transfer to naive mice, we examined the effect of CT on surface marker expression on immature DC and compared these effects with those induced with LPS. Incubation of DC with LPS for 24 h

enhanced the expression of CD80, CD86, OX40, and CD40 and decreased the expression of CCR5 (Fig. 9). Stimulation of immature DC with CT also clearly enhanced the expression of CD80, reduced the expression of CCR5, and moderately enhanced the expression of CD86 and OX40. However, unlike LPS, CT downregulated ICAM-1 and CD40 expression on DC (Fig. 9). Similar findings were obtained with DC from both C3H/HeN and LPS-hyporesponsive C3H/HeJ mice, suggesting that the modulation of surface markers observed with CT was not due to LPS (data not shown).

We also investigated the ability of CT to modulate LPS-induced DC maturation. Incubation of DC with CT suppressed LPS-driven up-regulation of CD40 and ICAM-1 (Fig. 9). The expression of CD86 was slightly reduced compared with that of cells treated
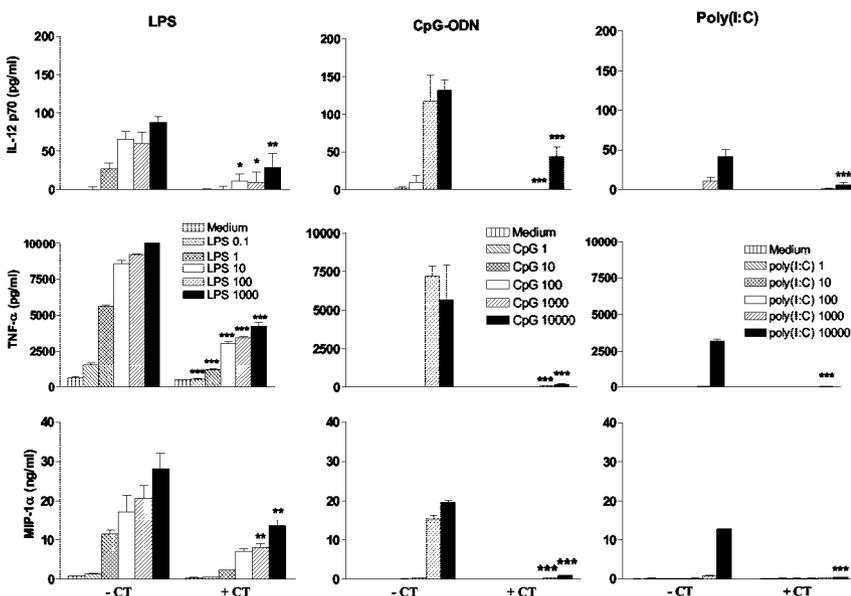
FIGURE 7. CT inhibits CpG, poly(I:C), and LPS induction of inflammatory cytokines and chemokines. DC (1×10^6/ml) were incubated for 6 h with medium alone, LPS (0.1–1000 ng/ml), CpG-ODN (1–10,000 ng/ml), or poly(I:C) (1–10,000 ng/ml) in the presence or the absence of CT (1 μg/ml). Supernatants were assayed for cytokines by immunoassays. Results are representative of five experiments. $*$, $p < 0.05$; $**$, $p < 0.01$; $***$, $p < 0.001$ (with vs without CT).

FIGURE 8. Inhibition of LPS-induced IL-12p40 and TNF-α mRNA expression in DC following cotreatment with CT. DC were treated with medium, CT (1 μg/ml), LPS (10 ng/ml), or CT and LPS for 1–12 h, and IL-12p40 and TNF-α mRNA were detected by RT-PCR. Agarose gels are representative of three replicates per experiment.

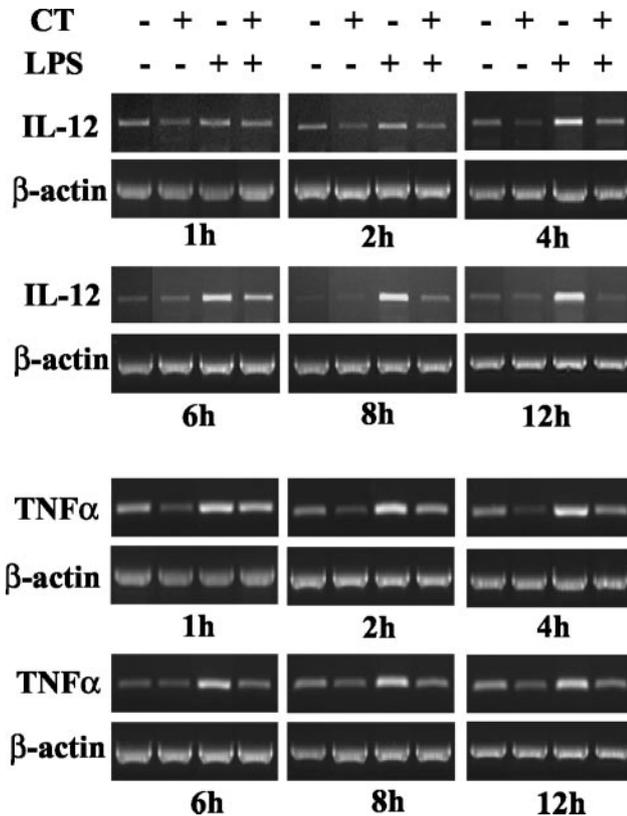**FIGURE 9.** CT enhances CD80 and CD86, but inhibits CD40 and ICAM-1 expression on DC. DC were stimulated with medium alone, CT (1 μg/ml), LPS (10 ng/ml), or CT plus LPS in the presence or the absence of anti-IL-10 (10 μg/ml). After 24-h incubation, cells were washed and stained with Abs specific for CD80, CD86, OX40, CCR5, ICAM-1, and CD40 or with isotype-matched control Abs. Immunofluorescence is shown for treated (black line) compared with untreated (gray histograms) DC. The numbers on the *right* of each histogram refer to the mean fluorescence intensity of the treated cells; the value for cells treated with medium alone is presented on the *left* of the first histogram in each case. Profiles are shown for a single experiment and are representative of five experiments.

with LPS alone. The expression of CCR5 was similar in cells treated with CT and LPS or with LPS alone. These effects were observed when DC were either preincubated with CT for 2 h before the addition of LPS (data not shown) or simultaneously exposed to CT and LPS (Fig. 9). We also observed a dramatic inhibitory effect of CT on poly(I:C)-stimulated expression of ICAM-1 and CD40, whereas the expression of CD80 and OX40 was strongly increased by the presence of CT (data not shown). These data indicate that CT induces DC maturation in a way strikingly different to TLR ligands, particularly in relation to the expression of CD40 and ICAM-1, and suggest that CT can modulate the activation of DC that are known to direct the induction of Th1 cells.

The inhibitory effect of CT on LPS-induced CD40 and ICAM-I expression was not reversed with an anti-IL-10 Ab (Fig. 9). In contrast, anti-IL-10 enhanced the expression of CD80 and CD86, but inhibited the expression of OX40 on DC treated with CT and LPS (Fig. 9). Anti-IL-10 also inhibited the expression of OX40 in response to CT or LPS alone (data not shown). This suggests that IL-10 does not mediate the inhibitory effect of CT on CD40 and ICAM-1 expression, but autocrine IL-10 or IL-10 induced by CT and LPS does have a suppressive effect on CD80 and CD86 expression, while promoting OX40 expression.

Discussion

A significant new finding of this study is that it is possible to generate Ag-specific T cells with regulatory activity by immunization with Ag in the presence of a pathogen-derived immunomodulatory molecule with adjuvant activity. Furthermore, we demonstrated that DC, which direct the induction of these Tr cells,
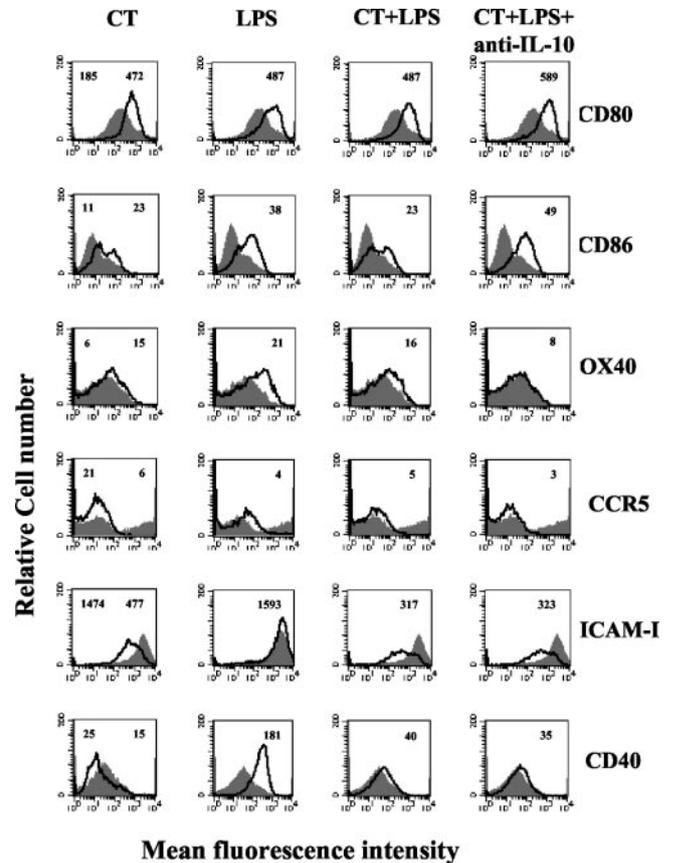
are not immature, but have distinct activation status from DC that drive Th1 cells. It has previously been demonstrated that Tr1-type cells can be generated in vitro by repeated Ag stimulation of T cells from TCR transgenic mice in the presence of IL-10 (7). Furthermore, Tr1 clones have been established from mice and humans infected with pathogens that cause persistent or chronic infection (4–6). However, Tr cells are difficult to propagate in vitro, and their therapeutic potential is dependent on the identification of immunization approaches for induction or activation of these cells in vivo.

The adjuvant activity of AB-type toxins is well documented, and the ability of CT, LT, and pertussis toxin to enhance adaptive immune responses to bystander Ag appears to be associated in part with their capacity to activate cells of the innate immune system. CT enhances the costimulatory potential of DC via the up-regulation of CD80 and CD86 expression, but also appears to modulate DC to preferentially activate IL-10-secreting T cells. Cytokines produced by cells of the innate immune system or by T cells have been implicated in the selective activation of Th1 and Th2 cells in vivo (8). A number of pathogens and pathogen-derived molecules that stimulate IL-12 production by macrophages and DC enhance the induction of Th1 responses (9, 10). In contrast, IL-4 as well as

IL-6 and IL-10 have been implicated in promoting the differentiation of Th2 cells (21, 22). More recently, it has been suggested that IL-10, TGF-β, and IL-4 may play a role in driving the differentiation of Tr cells (3, 23, 24). We found that CT, which was free of LPS, failed to induce any of these cytokines from DC in vitro. However, in the presence of low doses of LPS, CT induced the transcription and translation of IL-10. Furthermore, CT stimulated MIP-2 mRNA and protein in the absence of additional signals. Our preliminary experiments are consistent with a report demonstrating that MIP-2 gene expression in response to hydrogen peroxide was mediated through activation of cAMP (25). MIP-2 is thought to be involved in the recruitment of neutrophils, monocytes, and T cells, and we have found that recombinant MIP-2 can enhance immune responses to coadministered Ags (unpublished observations). Therefore, MIP-2 production in vivo may contribute to the adjuvant effect of CT.

In contrast to the positive effect on MIP-2 production, CT inhibited the production of the proinflammatory cytokines, IL-12 and TNF-α, and inflammatory chemokines, MIP-1α, MIP-1β, and MCP-1, induced in response to the TLR ligands, LPS, CpG-ODN, and poly(I:C). The synergy between LPS and CT in the induction of IL-10 production suggests that two signals may be required for CT to activate or enhance the production of certain cytokines from DC in vitro. However, the adjuvant effect of CT for the induction of IL-10-secreting T cells in vivo was not dependent on coadministration of LPS. It is possible that in vivo the second signal was provided by endogenous TLR ligands or activated T cells, since CT is also known to exert direct effects on T cells (26). DC have a major influence on the priming of naive T cells, and it has been suggested that plasmacytoid and myeloid DC promote Th1 and Th2 cells, respectively (27), whereas immature DC have been implicated in driving anergic or Tr cells (28). Alternatively, the same subtype of DC may selectively enhance the development of distinct T cell subtypes depending on the dose and type of Ag or immunomodulatory molecules and the environment pertaining at the time of maturation (2, 10, 29). Our data suggest that DC activated with CT promote the induction of IL-10-secreting T cells, but not Th1 cells. Adoptive transfer of Ag-pulsed CT-modulated DC induced T cells that secreted IL-10 and IL-4. Furthermore, Ag-specific T cell clones generated from mice immunized with Ag in the presence of CT secreted IL-10, IL-5, and IL-13 and varying concentrations of IL-4. It has previously been reported that Tr1 cells secrete high levels of IL-10, but, in addition, may secrete IL-5, but low or undetectable IL-4 (4–7). Our data demonstrate that T cells with regulatory activity can also secrete IL-13. IL-13 correlated with IL-5 production, and this is consistent with the demonstration that cAMP induced IL-5 and IL-13, but not IL-4, in Th2 cells via p38 kinase-induced phosphorylation of the transcriptional factor GATA-3 (30). It has been argued that Tr1 cells may arise from conventional Th2 cells that have lost their ability to produce IL-4, but retain their ability to secrete IL-10 (24). Our data demonstrate that T cells generated with CT as an adjuvant secrete high levels of IL-10 and are capable of suppressing IFN-γ production by Th1 cells and therefore fulfill the primary criteria for designation as Tr cells. Furthermore, the adoptive transfer experiments suggest that CT-modulated DC that direct the induction of IL-10-secreting T cells have a distinct phenotype from CpG-ODN- or LPS-stimulated DC, which drive Th1 cells (9, 10).

CT, LT, and pertussis toxin promote partial or complete maturation of DC, either alone or in the presence of other maturation stimuli, such as LPS or IL-1 and TNF-α (10, 12; our unpublished observations). Consistent with these findings, we demonstrated that stimulation of DC with CT enhanced the surface expression of CD80 and, to a lesser extent, that of CD86 and OX40 and reduced the surface expression of CCR5. However, we also report that CT inhibited the expression of CD40 and ICAM-1, whereas Th1-promoting molecules enhanced the expression of these markers. It was recently reported that DC that lack surface expression of CD40 suppressed a primed immune response and induced IL-10-secreting CD4$^+$ Tr cells (31). The results suggested that it was not DC maturity, but, rather, CD40 expression, that dictated the consequences of Ag presentation by myeloid DC. Furthermore, the ICAM-1-LFA-1 interaction is thought to promote the induction of Th1 cells independently of IL-12 (32). Thus, CT-activated DC, in which CD40 and ICAM-1 expression is suppressed, but CD80, CD86, and OX40 expression is enhanced, may promote the induction of Tr1 and Th2 cells, while blocking Th1 differentiation. While we do not suggest that the CT-treated DC are analogous to the DC specifically lacking CD40 expression (31), in that the CT-pulsed DC do drive an effector immune response, the down-regulation of CD40 by the toxin may contribute to the Tr1 cell induction.

Although both IL-10 production and inhibition of proinflammatory cytokines by CT and LT appears to involve ADP-ribosyltransferase-mediated activation of cAMP (18; our unpublished observations), the inhibition of proinflammatory cytokine/chemokine production and CD40 and ICAM-1 expression on DC does not appear to be primarily IL-10 dependent. We found that a neutralizing anti-IL-10 Ab had little or no ability to abrogate the inhibitory effects. This is consistent with a previous report on IL-12 inhibition (17) and our finding that the inhibition of LPS-induced TNF-α mRNA by CT was observed at 1 h, whereas the induction of IL-10 protein in response to LPS and CT was not detected until 2 h after stimulation of DC.

Since proinflammatory cytokine production and surface marker expression on DC are mediated by molecules that signal through mitogen-activated protein kinase and NF-κB pathways, it is likely that CT may exert its suppressive effects by targeting these signaling pathways. Activation of cAMP and, as a consequence, PKA was found to inhibit the induction of a set of NF-κB-regulated genes by acting on the *trans*-activation domain of p65 (33). We have preliminary data that indicates that CT inhibits NF-κB transcriptional activity (unpublished observations). Inhibition of NF-κB has been suggested as a mechanism to prevent differentiation of DC or drive them into a phenotype that promotes the induction of Tr cells (30, 34). While certain immune response genes that are activated through NF-κB or mitogen-activated protein kinases, including IL-12, TNF-α, MIP-1α, MIP-1β, and CD40, are suppressed by CT, others, including CD80, MIP-2, and IL-10, are activated. This suggests that CT can activate or inhibit divergent TLR-mediated signaling pathways that mediate distinct aspects of DC maturation and cytokine/chemokine secretion.

We have demonstrated that CT can promote IL-10 production and suppress proinflammatory cytokines and chemokines, while retaining the capacity to activate DC that direct naive T cells to differentiate into regulatory T cells. This may represent a potent mechanism evolved by *V. cholerae* to subvert protective Th1 responses and may explain certain in vivo immunomodulatory effects of AB toxins reported to date. CT is a powerful mucosal adjuvant, and preliminary experiments have revealed that nasal administration of Ags with adjuvants that enhance IL-10 production from DC promote the generation of IL-10-secreting T cells in vivo (our unpublished observations). Although CT itself is not suitable for use in humans, derivatives or molecules that mimic its immunomodulatory function without its toxicity have considerable potential in the development of immunotherapeutics for the treatment of immune-mediated diseases in which inflammatory Th1 responses play a role in pathology.

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