

Effects of cholera toxin on innate and adaptive immunity and its application as an immunomodulatory agent

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Abstract: Cholera toxin (CT) is a potent vaccine adjuvant when administered via parenteral, mucosal, or transcutaneous routes. It also inhibits innate inflammatory responses induced by pathogen-derived molecules, such as lipopolysaccharide (LPS). We demonstrated previously that CT promotes the induction of regulatory type 1 T cells (Tr1) as well as T helper type 2 cells (Th2). T cells from mice immunized with antigen in the presence of CT produced high levels of interleukin (IL)-10 and IL-5 and low levels of IL-4 and interferon- γ (IFN- γ). Here, we demonstrate that immunization with antigen in the presence of CT induced a population of antigen-specific CD4⁺ T cells that produced IL-10 in the absence of IL-4, in addition to cells that coexpressed IL-4 and IL-10 or produced IL-4 only. CT-generated Tr1 cells inhibited antigen-specific proliferation as well as IFN- γ production by Th1 cells, and this suppression was cell contact-independent. It is interesting that coincubation with Th1 cells significantly enhanced IL-10 production by the Tr1 cells. As IL-10 can promote the differentiation of Tr1 cells, we investigated cytokine production by dendritic cells (DC) following exposure to CT. Previous data showed that CT can modulate the expression of costimulatory molecules and inhibit the production of chemokines and cytokines, including IL-12 and tumor necrosis factor α and enhance IL-10 production. Here, we show that CT synergizes with LPS to induce IL-6 and IL-1 β in addition to IL-10 production by immature DC. Therefore, CT may promote the induction of Th2 and Tr1 cells in part via selective modulation of DC cytokine production and costimulatory molecule expression. *J. Leukoc. Biol.* 75: 756–763; 2004.

Key Words: regulatory T cell · dendritic cell · innate immunity · vaccination · tolerance/suppression/anergy · Th1/Th2 cell

INTRODUCTION

Cholera toxin (CT) from *Vibrio cholerae* is a member of the AB class of bacterial toxins. It is composed of an enzymatically active A subunit with adenosine-diphosphate (ADP)-ribosyl-

transferase activity that is responsible for toxicity and a pentameric B oligomer (CTB) that is necessary for internalization of the globular A subunit after binding to cell-surface receptors [1–6]. In the case of mucosal immunization, specific interaction of the B subunit with its receptor on epithelial cells is necessary for uptake from the lumen of the gastrointestinal or respiratory tract. Although CTB can bind to a number of galactose-containing molecules, it binds with high affinity to the glycosphingolipid, GM1-ganglioside [Gal(β 1-3)GalNAc(β 1-4)(NeuAc(α 2-3))Gal(β 1-4)Glc(β 1-1)ceramide; ref. 7]. CTB also binds to GD1b-ganglioside but with a lower affinity [5]. Recent evidence indicates that the adjuvant activity of CT and its ability to activate dendritic cells (DC) are dependent on its specific interaction with GM1 ganglioside [8].

The CT A subunit is composed of a globular A₁ domain and an A₂ domain that interacts with the B subunit. The ADP-ribosyltransferase activity is facilitated following proteolytic cleavage of the trypsin-sensitive loop between the two domains and reduction of the disulphide bond [7]. The A₁ fragment enters the cytosol, and ADP ribosylates the stimulatory α subunit of a guanosine 5'-triphosphate (GTP)-binding protein (G_s) that causes permanent activation of the adenylate cyclase, resulting in an elevation in intracellular cyclic adenosine monophosphate (cAMP) concentration [6, 9]. The C terminus of the A₂ fragment contains a sequence motif associated with retrieval of proteins from the trans-Golgi network to the endoplasmic reticulum [10]. This may be important in delivering the A₁ fragment to the correct cellular compartment [4]. The basal ADP-ribosyltransferase activity of the toxin is enhanced by interaction with GTP-binding proteins, known as ADP-ribosylation factors [11]. These factors play a crucial role in vesicular membrane trafficking and contribute to the maintenance of organelle integrity and the assembly of coat proteins.

IMMUNOGENICITY AND ADJUVANTICITY OF CT

CT is a powerful parenteral and mucosal immunogen; low doses of the toxin can induce strong antitoxin, secretory, and

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systemic antibody responses [12]. In addition, immunization with antigen in the presence of CT via parenteral, mucosal, and transcutaneous routes results in substantial enhancement of mucosal immunoglobulin A (IgA) and serum IgG responses to the coadministered antigen [4]. CT also activates cellular immune responses to coadministered antigens and enhances the induction of CD4⁺ T helper and class I-restricted cytolytic T lymphocyte responses [13–15]. Most studies indicate that CT promotes a strong T helper cell type 2 (Th2)-biased response to itself and to bystander antigens. This conclusion is based on T cell production of interleukin (IL)-4, IL-5, and IL-10 with little interferon- γ (IFN- γ) [16–21] and is supported by evidence that IgE [16, 18, 22] and higher titers of IgG1 than IgG2a [16, 18, 21–27] are induced after immunization with antigens in the presence of CT. However, other studies have reported mixed Th1/Th2 (with the production of IFN- γ and IL-4) responses following oral [28–30] and intranasal immunization [31] with antigens in the presence of CT. Our data indicate that although some IFN- γ is produced, the response is Th2-biased, but in addition, CT induces a population of IL-10-producing T cells that have regulatory activity.

INDUCTION OF ANTIGEN-SPECIFIC REGULATORY T CELLS BY CT

Although CT is able to activate the production of a number of cytokines associated with Th2 and to a lesser degree, Th1 cells, it also induces a population of IL-10-producing T cells with suppressor activity. T cell lines established from the spleens of mice immunized with antigens in the presence of CT secreted variable concentrations of the cytokines IL-4, IL-5, and IL-10. Antigen-specific T cell clones established from these lines included clones that produced IL-10 in the absence of IL-4 [32]. This is consistent with a number of studies that have reported that type 1 T regulatory (Tr1) cells secrete high levels of IL-10 but in addition, may also secrete IL-5, with low or undetectable IL-4 [33, 34]. To demonstrate that distinct populations of antigen-specific IL-4- and IL-10-secreting T cells were generated in vivo, intracellular staining was performed on T cells from spleens of mice immunized subcutaneously with antigen in the presence or absence of CT (**Fig. 1**). Immuniza-

tion of mice with KLH alone or in the presence of CT resulted in the induction of CD4⁺ T cells producing IL-4 and IL-10. However, there was a considerable increase in the percentage of IL-10-producing CD4⁺ T cells in the mice immunized with KLH in the presence of CT. Although some of these CD4⁺ T cells produced IL-4 and IL-10, there was a large population of CD4⁺ T cells that produced IL-10, independently of IL-4. The phenotype and ontogeny of these IL-10-producing, antigen-specific CD4⁺ T cells are uncertain at present, but these may represent a distinct T cell population. Tr1 cells may arise from naïve cells in lymph nodes following presentation by DC, in which IL-10 production is enhanced, and IL-12 production is inhibited [32, 33]. Alternatively, Tr1 cells may be derived from conventional Th2 cells that have lost their ability to produce IL-4 but retain their ability to secrete IL-10 [35]. In addition to our evidence that parenteral immunization with the CT holotoxin can generate a population of Tr1 cells, mucosal immunization with CTB-antigen conjugates induces regulatory T cells capable of suppressing autoimmune diseases mediated by Th1 cells [36, 37].

Experiments were performed to determine whether the antigen-specific, IL-10-producing Tr1 cells induced in the presence of CT could exhibit regulatory activity against Th1 cells. A Th1 cell line was generated from spleens of mice immunized with KLH in the presence of the Toll-like receptor 9 (TLR9) ligand, CpG oligodeoxynucleotide (CpG). These T cells proliferated strongly and produced high levels of IFN- γ when restimulated with KLH (**Fig. 1**). In contrast, the CT-generated Tr1 cells proliferated very poorly and produced no IFN- γ . This concurs with reports that under these conditions, CD4⁺CD25⁺ Tr cells are difficult to expand in vitro [38]. However, recent data suggest that in contrast to reports of anergy in vitro, CD4⁺CD25⁺ Tr cells can proliferate in response to antigen in vivo [39]. Furthermore, CD4⁺CD25⁺ Tr cells are able to expand on incubation with antigen-loaded, mature DC in vitro [40]. The poor proliferation of the CT-generated Tr1 cells may also be an in vitro phenomenon related to the type of antigen-presenting cell (APC), the absence of growth factors, or the presence of suppressive cytokines such as IL-10. Coincubation of Th1 with Tr1 cells resulted in a significant suppression of Th1 cell proliferation, although this was only achieved at a high ratio of Tr1-to-Th1 cells (**Fig. 2**). Furthermore, a Tr1-

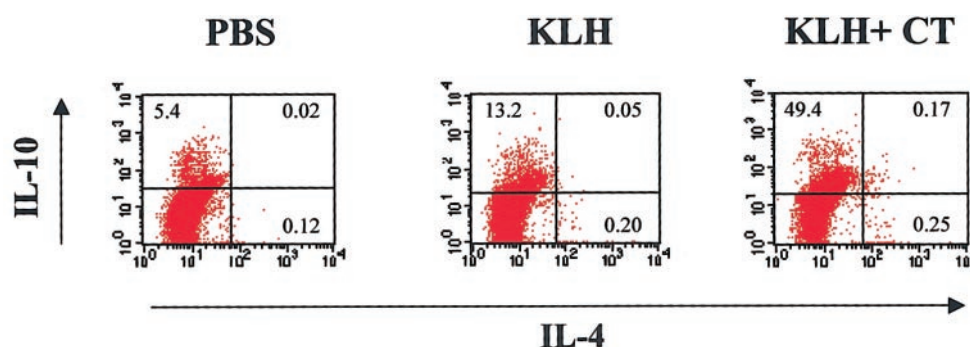


Fig. 1. CT induces IL-10-producing CD4⁺ Tr1 cells with a regulatory phenotype. Single spleen-cell suspensions from mice immunized with phosphate-buffered saline (PBS), keyhole limpet hemocyanin (KLH), or KLH + CT were stimulated with KLH (10 μ g/ml) for 3 days. Cells were then further stimulated for 6 h with phorbol 12-myristate 13-acetate (10 ng/ml) and ionomycin (1 μ g/ml). Brefeldin A (10 μ g/ml) was added for the final 4 h, after which cells were washed and blocked with 50% fetal calf serum. Surface and intracellular staining was per-

formed using the Caltag Fix and Perm Cell permeabilization kit (GAS003, Caltag Laboratories, South San Francisco, CA). Cells were surface-stained with rat anti-mouse CD4 and intracellularly, with rat anti-mouse IL-4 and IL-10. All antibodies were purchased from Caltag Laboratories. Staining was assessed by FACSCalibur (BD Biosciences, San Jose, CA), and data were analyzed using CellQuest software for MacIntosh.

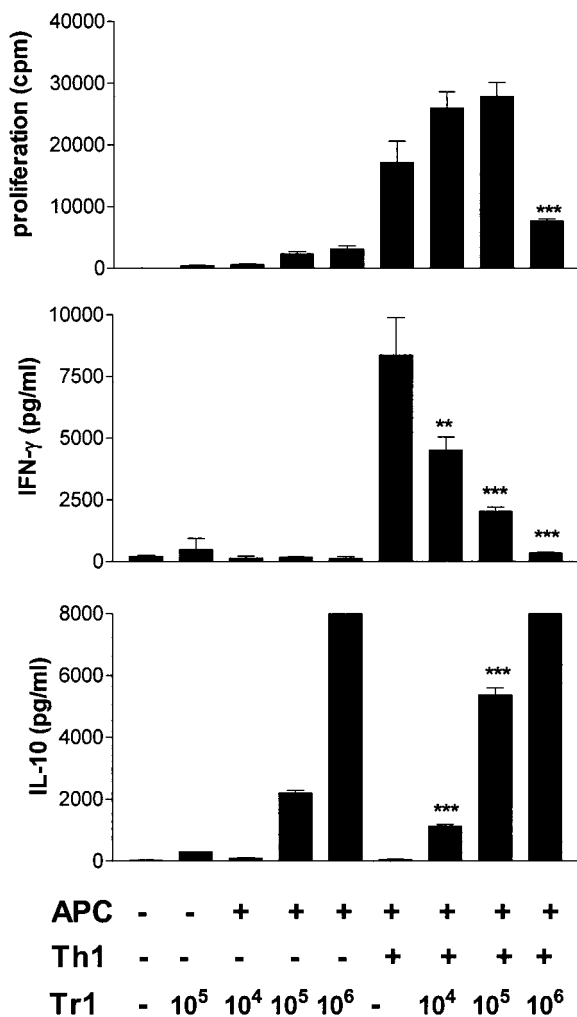


Fig. 2. Immunization with antigen in the presence of CT induces Tr1 cells that suppress proliferation and IFN- γ production by Th1 cells. A KLH-specific, Tr1-type T cell line (1×10^4 – 1×10^6 /ml) generated from mice immunized with KLH in the presence of CT and a KLH-specific Th1 cell line (1×10^5 /ml) generated from mice immunized with KLH in the presence of CpG were cultured alone or together (separated via a semipermeable membrane) in the presence of APC (irradiated syngeneic spleen cells; 2×10^6 /ml) and KLH (50 μ g/ml). Supernatants were collected after 72 h for analysis of IFN- γ and IL-10 and replaced with fresh medium, 3 H-thymidine was added, and cells were incubated at 37° for 4 h. Cells were harvested, and proliferation was determined by measuring thymidine incorporation. Results are means ($\pm$ SD) for triplicate cultures. **, $P < 0.01$; ***, $P < 0.001$, Th1 versus Th1 + Tr1 at the same Th1 cell number. Cytokine concentrations 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.

induced inhibition of IFN- γ production by the Th1 cells was observed (Fig. 2). The inhibition of Th1 cell proliferation and IFN- γ production did not require cell-to-cell contact, as suppression occurred when cells were separated with a semipermeable membrane. The Tr1 cells produced high levels of IL-10 on stimulation with KLH, and this was significantly enhanced (at the lower Tr1:Th1 cell ratios) when the Tr1 cells were coincubated with Th1 cells (Fig. 2). This effect was not cell contact-dependent and indicates that the suppressive activity

of the Tr1 cells may be up-regulated in a controlled manner on contact with inflammatory Th1 cells.

ROLE OF DC IN CT-INDUCED IMMUNE RESPONSES

The mechanism used by CT to promote the induction of specific T cell subtypes *in vivo* has not been fully elucidated but is likely to involve direct interactions with APC and lymphocytes. DC are pivotal in the initiation of T cell responses and in the instruction of antigen-specific, naïve T cells [41]. It has been suggested that plasmacytoid and myeloid DC promote Th1 and Th2 cell responses, respectively [42], whereas immature DC have been implicated in the induction of anergic or Tr cells, partly through the lack of costimulation signals and consequent downstream effects [41]. Alternatively, the same subtype of DC may selectively enhance the development of distinct T cell subtypes, depending on the dose and type of antigen or immunomodulatory molecules and the environment pertaining at the time of maturation [32, 33, 43–45]. Adoptive-transfer experiments have also shown that modulation of DC with particular pathogen-derived molecules can induce polarized Th1 or Th2 responses *in vivo* [32, 46]. We demonstrated that DC pulsed with antigen in the presence of CT induced a Tr1/Th2 response *in vivo*, characterized by high levels of antigen-specific IL-10 [32]. This indicates that direct effects of CT on DC can at least partly explain its immunological actions.

CT can promote DC maturation alone or in the presence of additional stimuli such as lipopolysaccharide (LPS) or a combination of IL-1 and tumor necrosis factor α (TNF- α) [44, 45]. We have previously found that exposure of DC to CT enhanced surface expression of CD80 and to a lesser extent, CD86 and OX40 and reduced the surface expression of the chemokine receptor CCR5 as well as CD40 and intercellular adhesion molecule-1 (ICAM-1; **Table 1**) [32]. This contrasts with Th1-promoting molecules such as CpG, polyinosinic-polycytidylic acid (poly I:C), and LPS, which enhance expression of each of these surface-expressed molecules [43, 44]. Costimulatory molecule expression plays an important role in the ability of DC to promote distinct T cell responses. It has been suggested that expression of major histocompatibility complex (MHC) class II, CD80, and CD86 can influence the ability of DC to direct naïve T cells into Th1 or Th2 subtypes [47] and that the primary response was strictly dependent on these interactions. As CT does not inhibit MHC class II expression and enhances CD80 and CD86 expression on DC, this may fulfill the primary requirements for the DC to activate T cells. Additionally, selective inhibition of the expression of costimulatory molecules such as CD40 and ICAM-1 may play a role in the polarization of Tr1/Th2 responses by the toxin. Ineffective CD40 ligation has been associated with T cell unresponsiveness and reduced type 1 cytokines but enhanced IL-10 production [48, 49]. Thus, the ability of CT to selectively modulate DC costimulatory molecule expression may promote the induction of Tr1 and Th2 cells and block Th1 differentiation.

TABLE 1. Summary of the Effects of CT on DC Cytokine, Chemokine, and Costimulatory Molecule Expression

	Induced	Enhanced	Inhibited
Cytokines			
MIP-2	+		
IL-1 β		+	
IL-6		+	
IL-10		+	
IL-12 p70			+
IL-12 p40			+
TNF- α			+
MIP-1 α			+
MIP-1 β			+
MCP-1			+
Costimulatory/surface-expressed molecules			
CD80	+	+	
CD86	+		
OX40	+	+	
CD14	+		
CD40			+
ICAM-1			+

The columns refer to the direct effects of CT (Induced) or modulation of LPS-mediated effects (Enhanced/Inhibited) on DC. CT was added to DC at 1 μ g/ml alone or 1 h prior to addition of *Escherichia coli* LPS (0.1–10 ng/ml). Cytokine expression was determined by enzyme-linked immunosorbent assay (ELISA) and costimulatory molecule expression by flow cytometry. MIP-2, macrophage-inflammatory protein-2; MCP-1, monocyte chemoattractant protein-1.

Although the polarization of naïve T cells is multifactorial, the cytokine environment pertaining at the time of primary activation appears central [50]. A number of cytokines produced by cells of the innate-immune system and/or T cells are involved in the selective activation of Th1 and Th2 cells [51]. Secretion of IL-12 by DC and macrophages enhances Th1 responses [43]. In contrast, IL-4 as well as IL-6 and IL-10 can promote the differentiation of Th2 cells [52, 53]. Indeed, IL-10 was required for optimal generation of Th2 cells by CD8 α^+ DC [54]. IL-10, IL-4, and transforming growth factor- β (TGF- β) may be involved in driving the differentiation of Tr cells [35, 55, 56]. We were unable to detect the production of IL-6, IL-4, or IL-10 by DC exposed to CT alone in vitro. However, in the presence of low doses of LPS, CT significantly enhanced IL-10 production (**Fig. 3**) [32]. In addition, CT synergized with LPS to enhance the production of IL-6 and IL-1 β (Fig. 3). The enhancement of IL-6 was only evident with low concentrations of LPS, and IL-10 and IL-1 β production was enhanced over a wide range of LPS concentrations (Fig. 3). The enhancement of LPS-mediated IL-6 production by CT was also detected at the transcriptional level (data not shown).

Enhancement of LPS-induced IL-6 and IL-1 β production by CT was previously demonstrated in murine macrophages [57]. IL-6 can promote Th2 differentiation via the activation of nuclear factor of activated T cells and induction of early IL-4 production by CD4 $^+$ T cells [58] and can inhibit IFN- γ production and Th1 differentiation. Following intranasal immunization, antigen-loaded, pulmonary DC produced IL-6 and IL-10, which was proposed to promote Th2 differentiation in situ

[59]. The propensity of the lung microenvironment to generate Th2-dominated responses thus appears to be associated with IL-10 and IL-6 induction. However, these cytokines may also induce the differentiation of T cells into Tr cells [33]. There is some evidence that signaling through the IL-1 receptor 1 may also promote Th2 responses [60]. As much of the work on cytokine-mediated differentiation of Th cells did not attempt to separate Th2 from Tr1 responses, it is presently unclear whether factors that drive Th2 differentiation also play a role in promoting Tr1 responses.

In contrast to the up-regulated secretion of the above cytokines, CT inhibited the production of the proinflammatory cytokines IL-12 and TNF- α and inflammatory chemokines MIP-1 α , MIP-1 β , and MCP-1, induced in response to the TLR ligands LPS (Table 1), CpG, and poly I:C [32]. In human DC,

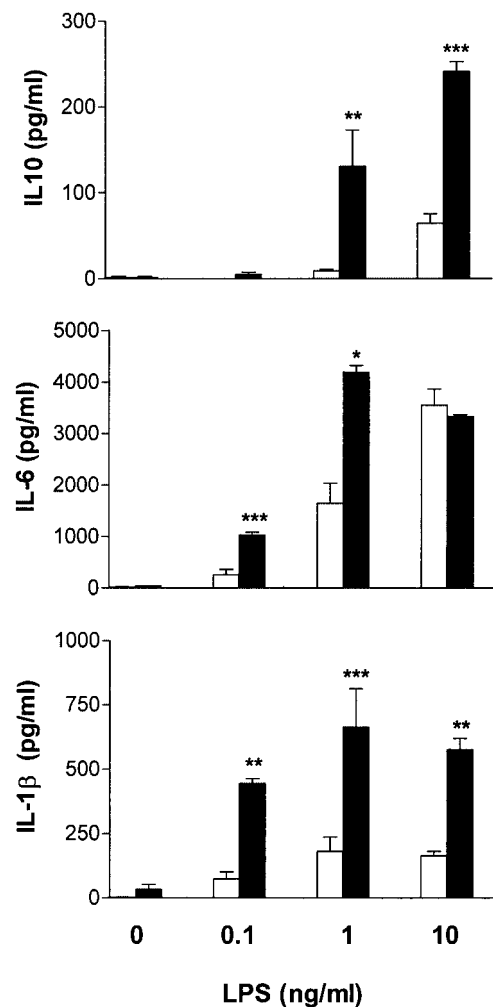


Fig. 3. CT synergizes with LPS to induce IL-10, IL-6, and IL-1 β production by DC. Bone marrow-derived, immature DC were incubated for 12 h with LPS (0.1–10 ng/ml) in the presence (■) or absence (□) of CT (1 μ g/ml) or with CT alone. Cytokine concentrations were determined in supernatants by ELISA. Results are means ($\pm$ SD) of triplicate assays and are representative of four experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, CT + LPS versus LPS at the same concentration. Cytokine concentrations 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.

CT inhibited the production of IL-12 and the expression of IL-12 receptor chains and TNF- α , leading to a suppression of Th1 cell differentiation [45, 61]. Forskolin, a pharmacological activator of adenylate cyclase, can also inhibit the production of bioactive IL-12, TNF- α , and MIP-1 α and can enhance IL-10 production by DC, implicating cAMP as a determining factor in the inhibitory action of CT on inflammatory cytokines (E. C. Lavelle et al., unpublished data).

Although our recent studies point to a dominant role for the elevation in intracellular cAMP concentrations in the inhibitory effects of CT, other workers have found that CTB can also modulate DC activation. Pretreatment of monocytes and macrophages with relatively high concentrations of CTB reduced their subsequent responsiveness to LPS [62]. LPS-induced production of TNF- α , IL-6, IL-12 p70, and nitric oxide was inhibited, and IL-10 production was enhanced. This suggests that in addition to the cAMP-mediated enhancement of LPS-driven IL-10 reported by our group and others, the B subunit may also enhance IL-10 production by cells of the innate-immune system. However, the inhibitory effect of CTB on macrophage IL-6 production is in contrast to the enhanced effect of CT on IL-6 production by macrophages [57] and DC. Antibodies to IL-10 and TGF- β prevented the inhibitory effect of CTB on LPS-induced IL-6 and TNF- α production [62]. However, the effect of anti-IL-10 and TGF- β antibodies on IL-6 production was greater than on TNF- α production, indicating a role for additional factors in the inhibition of TNF- α by CTB.

CT AS A POTENTIAL THERAPEUTIC FOR AUTOIMMUNE DISEASES

The anti-inflammatory effects of CT and its ability to promote the induction of antigen-specific Tr cells underline its potential for the treatment of diseases mediated by Th1 cells. It has been reported that nasal administration of high doses of CT can suppress clinical signs of experimental autoimmune encephalomyelitis (EAE), a murine model of multiple sclerosis [63]. The concentrations of IFN- γ and IL-12 in the central nervous system (CNS) of CT-treated mice were lower than in controls. CT was also shown to potentiate tolerance to bovine peripheral nerve myelin in the experimental autoimmune neuritis model of human inflammatory demyelinating neuropathies [64]. There is also earlier evidence that CT could induce tolerance to allografts in mice [65]. Thus, in a number of T cell-mediated, autoimmune conditions, mucosal administration of CT has been shown to enhance the induction of tolerance and alleviate disease symptoms.

Although it is conceptually attractive to attribute the anti-inflammatory effects of CT to the elevated cAMP levels mediated by the A subunit, a large body of evidence now exists indicating that mucosal delivery of the CTB subunit can independently exert anti-inflammatory effects. Oral delivery of CTB conjugated to myelin basic protein protected mice against the development of EAE [66, 67]. It was proposed that the inhibitory effect was a result of the induction of TGF- β -producing Tr

cells and down-regulation of chemokines in the CNS. However, the inclusion of CT in this system abrogated the protective effects of CTB. Antigen-CTB conjugates have also recently been shown to be protective in a number of other autoimmune models. Oral delivery of CTB conjugated to a heat-shock protein 60 kDa-derived peptide prevented mucosally induced uveitis in rats, an effect that was associated with enhanced IL-10 and TGF- β and reduced IL-12 and IFN- γ production [36]. Furthermore, oral administration of a CTB-insulin conjugate prevented diabetes in nonobese diabetic mice, which was associated with a reduction in IFN- γ production and Tr cell migration into pancreatic islets [68]. Oral administration of recombinant CTB also prevented IL-12-mediated murine experimental trinitrobenzene sulfonic acid-induced colitis [69]. Decreased IL-12 and IFN- γ production was documented, but in this case, there was no elevation in IL-10 or TGF- β . CTB conjugates were also effective in the induction of tolerance to type II collagen, leading to a suppression of chondritis in a model of autoimmune ear disease [70]. Oral administration of allogeneic antigen linked to CTB induced, immunological tolerance against allograft rejection [66]. It was shown that even without conjugation, CTB could potentiate oral tolerance induction to insulin [71]. In addition to this work on CTB, there is extensive evidence that the related B subunit of *E. coli* heat-labile enterotoxin can potentiate mucosal tolerance and prevent the induction of autoimmune inflammatory conditions including collagen-induced arthritis [4, 72]. Thus, it appears that the A-B holotoxins and their purified B chains have potential in treatment of inflammatory conditions via their anti-inflammatory properties and through the induction of Tr cells.

CONCLUSIONS

CT is a powerful mucosal adjuvant but also has potential as an immunomodulatory and anti-inflammatory agent. We recently demonstrated that in addition to its well-documented Th2-promoting activity, CT enhances the induction of a population of IL-10-producing Tr cells. Adoptive transfer of myeloid DC pulsed with antigen in the presence of CT can induce antigen-specific T cells with a similar cytokine profile to that observed following direct immunization [32], indicating that the adjuvant and modulatory activities of the toxin in vivo are at least partly attributable to its effects on DC. CT can enhance the expression of a number of costimulatory molecules on DC but in contrast to Th1-driving molecules such as CpG and poly I:C, inhibited the expression of CD40 and ICAM-1. In addition, in vitro studies with macrophages and DC have shown that CT can enhance the secretion of IL-10, IL-6, and IL-1 β in the presence of limiting doses of LPS. These selective inhibitory and synergistic effects of CT on DC may explain the ability of CT to promote the induction of antigen-specific Tr1 and Th2 cells (Fig. 4). The use of CT and *E. coli* heat-labile enterotoxin (LT) as therapeutic agents is hampered by their toxicity, and it is not yet completely clear which of the beneficial adjuvant and immunomodulatory activities are retained in nontoxic mutants

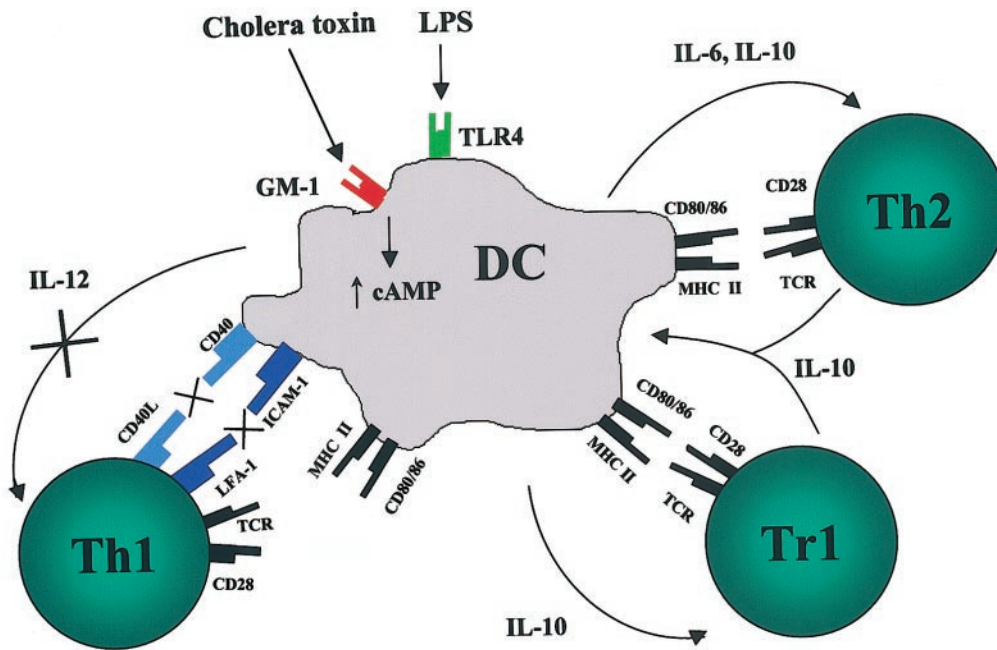


Fig. 4. Proposed model of the modulatory effects of CT on innate- and adaptive-immune responses. CT binds to GM-1 gangliosides on DC with high affinity, leading to entry of the toxin and enhancement of intracellular cAMP concentrations via the actions of the A subunit. These toxin-mediated effects selectively inhibit induction of the costimulatory molecules CD40 and ICAM-1, which play a role in Th1 cell differentiation. In contrast, the toxin enhances expression of other costimulatory molecules, particularly CD80 and CD86. In addition, CT inhibits the induction of IL-12, which is important in the differentiation of naive T cells toward a Th1 phenotype and in the presence of a second signal (e.g., LPS), enhances the production of other cytokines such as IL-10 and IL-6, which are important for Tr1/Th2 cell differentiation. TCR, T cell receptor; LFA-1, lymphocyte function-associated antigen-1.

or subunits. However, the B subunits and site-directed mutants of CT and LT, which are currently in clinical trials, have shown potential as adjuvants and therapeutics for a number of immune-mediated diseases.

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REFERENCES

- Zhang, R. G., Scott, D. L., Westbrook, M. L., Nance, S., Spangler, B. D., Shipley, G. G., Westbrook, E. M. (1995) The three-dimensional crystal structure of cholera toxin. *J. Mol. Biol.* **251**, 563–573.
- Rappuoli, R., Pizza, M., Douce, G., Dougan, G. (1999) Structure and mucosal adjuvant activity of cholera and *Escherichia coli* heat-labile enterotoxins. *Immunol. Today* **20**, 493–500.
- Pizza, M., Giuliani, M. M., Fontana, M. R., Monaci, E., Douce, G., Dougan, G., Mills, K. H., Rappuoli, R., Del Giudice, G. (2001) Mucosal vaccines: nontoxic derivatives of LT and CT as mucosal adjuvants. *Vaccine* **19**, 2534–2541.
- Williams, N. A., Hirst, T. R., Nashar, T. O. (1999) Immune modulation by the cholera-like enterotoxins: from adjuvant to therapeutic. *Immunol. Today* **20**, 95–101.
- Holmgren, J., Lonnroth, I., Svennerholm, L. (1973) Tissue receptor for cholera exotoxin: postulated structure from studies with GM1 ganglioside and related glycolipids. *Infect. Immun.* **8**, 208–214.
- Spangler, B. D. (1992) Structure and function of cholera toxin and the related *Escherichia coli* heat-labile enterotoxin. *Microbiol. Rev.* **56**, 622–647.
- Gill, D. M., Rappaport, R. S. (1979) Origin of the enzymatically active A1 fragment of cholera toxin. *J. Infect. Dis.* **139**, 674–680.
- Kawamura, Y. I., Kawashima, R., Shirai, Y., Kato, R., Hamabata, T., Yamamoto, M., Furukawa, K., Fujihashi, K., McGhee, J. R., Hayashi, H., Dohi, T. (2003) Cholera toxin activates dendritic cells through dependence on GM1-ganglioside which is mediated by NF- κ B translocation. *Eur. J. Immunol.* **33**, 3205–3212.
- Field, M., Rao, M. C., Chang, E. B. (1989) Intestinal electrolyte transport and diarrheal disease (2). *N. Engl. J. Med.* **321**, 879–883.
- Pelham, H. R. (1992) The Florey Lecture, 1992. The secretion of proteins by cells. *Proc. R. Soc. Lond. B. Biol. Sci.* **250**, 1–10.
- Tsai, S. C., Noda, M., Adamik, R., Chang, P. P., Chen, H. C., Moss, J., Vaughan, M. (1988) Stimulation of choleragen enzymatic activities by GTP and two soluble proteins purified from bovine brain. *J. Biol. Chem.* **263**, 1768–1772.
- Lavelle, E. C., Grant, G., Pusztai, A., Pfeller, U., O'Hagan, D. T. (2000) Mucosal immunogenicity of plant lectins in mice. *Immunology* **99**, 30–37.
- Simmons, C. P., Hussell, T., Sparer, T., Walz, G., Openshaw, P., Dougan, G. (2001) Mucosal delivery of a respiratory syncytial virus CTL peptide with enterotoxin-based adjuvants elicits protective, immunopathogenic, and immunoregulatory antiviral CD8⁺ T cell responses. *J. Immunol.* **166**, 1106–1113.
- Simmons, C. P., Mastroeni, P., Fowler, R., Ghaem-maghami, M., Lycke, N., Pizza, M., Rappuoli, R., Dougan, G. (1999) MHC class I-restricted cytotoxic lymphocyte responses induced by enterotoxin-based mucosal adjuvants. *J. Immunol.* **163**, 6502–6510.
- Bowen, J. C., Nair, S. K., Reddy, R., Rouse, B. T. (1994) Cholera toxin acts as a potent adjuvant for the induction of cytotoxic T-lymphocyte responses with non-replicating antigens. *Immunology* **81**, 338–342.
- Simecka, J. W., Jackson, R. J., Kiyono, H., McGhee, J. R. (2000) Mucosally induced immunoglobulin E-associated inflammation in the respiratory tract. *Infect. Immun.* **68**, 672–679.
- Yamamoto, M., Rennert, P., McGhee, J. R., Kweon, M. N., Yamamoto, S., Dohi, T., Otake, S., Bluethmann, H., Fujihashi, K., Kiyono, H. (2000) Alternate mucosal immune system: organized Peyer's patches are not required for IgA responses in the gastrointestinal tract. *J. Immunol.* **164**, 5184–5191.
- Yamamoto, S., Kiyono, H., Yamamoto, M., Imaoka, K., Fujihashi, K., Van Ginkel, F. W., Noda, M., Takeda, Y., McGhee, J. R. (1997) A nontoxic mutant of cholera toxin elicits Th2-type responses for enhanced mucosal immunity. *Proc. Natl. Acad. Sci. USA* **94**, 5267–5272.
- Yamamoto, S., Takeda, Y., Yamamoto, M., Kurazono, H., Imaoka, K., Fujihashi, K., Noda, M., Kiyono, H., McGhee, J. R. (1997) Mutants in the ADP-ribosyltransferase cleft of cholera toxin lack diarrheagenicity but retain adjuvant activity. *J. Exp. Med.* **185**, 1203–1210.
- Clarke, C. J., Wilson, A. D., Williams, N. A., Stokes, C. R. (1991) Mucosal priming of T-lymphocyte responses to fed protein antigens using cholera toxin as an adjuvant. *Immunology* **72**, 323–328.
- Xu-Amano, J., Kiyono, H., Jackson, R. J., Staats, H. F., Fujihashi, K., Burrows, P. D., Elson, C. O., Pillai, S., McGhee, J. R. (1993) Helper T cell subsets for immunoglobulin A responses: oral immunization with tetanus toxoid and cholera toxin as adjuvant selectively induces Th2 cells in mucosa associated tissues. *J. Exp. Med.* **178**, 1309–1320.

22. Marinaro, M., Staats, H. F., Hiroi, T., Jackson, R. J., Coste, M., Boyaka, P. N., Okahashi, N., Yamamoto, M., Kiyono, H., Bluethmann, H., et al. (1995) Mucosal adjuvant effect of cholera toxin in mice results from induction of T helper 2 (Th2) cells and IL-4. *J. Immunol.* **155**, 4621–4629.
23. Douce, G., Fontana, M., Pizza, M., Rappuoli, R., Dougan, G. (1997) Intranasal immunogenicity and adjuvanticity of site-directed mutant derivatives of cholera toxin. *Infect. Immun.* **65**, 2821–2828.
24. Pierre, P., Denis, O., Bazin, H., Mbongolo Mbella, E., Vaerman, J. P. (1992) Modulation of oral tolerance to ovalbumin by cholera toxin and its B subunit. *Eur. J. Immunol.* **22**, 3179–3182.
25. Glenn, G. M., Scharton-Kersten, T., Vassell, R., Mallett, C. P., Hale, T. L., Alving, C. R. (1998) Transcutaneous immunization with cholera toxin protects mice against lethal mucosal toxin challenge. *J. Immunol.* **161**, 3211–3214.
26. Ruedl, C., Rieser, C., Kofler, N., Wick, G., Wolf, H. (1996) Humoral and cellular immune responses in the murine respiratory tract following oral immunization with cholera toxin or *Escherichia coli* heat-labile enterotoxin. *Vaccine* **14**, 792–798.
27. Richards, C. M., Shimeld, C., Williams, N. A., Hill, T. J. (1998) Induction of mucosal immunity against herpes simplex virus type 1 in the mouse protects against ocular infection and establishment of latency. *J. Infect. Dis.* **177**, 1451–1457.
28. Hornquist, E., Lycke, N. (1993) Cholera toxin adjuvant greatly promotes antigen priming of T cells. *Eur. J. Immunol.* **23**, 2136–2143.
29. Pacheco, S. E., Gibbs, R. A., Ansari-Lari, A., Rogers, P. (2000) Intranasal immunization with HIV reverse transcriptase: effect of dose in the induction of helper T cell type 1 and 2 immunity. *AIDS Res. Hum. Retroviruses* **16**, 2009–2017.
30. Schaffeler, M. P., Brokenshire, J. S., Snider, D. P. (1997) Detection of precursor Th cells in mesenteric lymph nodes after oral immunization with protein antigen and cholera toxin. *Int. Immunol.* **9**, 1555–1562.
31. Yanagita, M., Hiroi, T., Kitagaki, N., Hamada, S., Ito, H. O., Shimauchi, H., Murakami, S., Okada, H., Kiyono, H. (1999) Nasopharyngeal-associated lymphoreticular tissue (NALT) immunity: fimbriae-specific Th1 and Th2 cell-regulated IgA responses for the inhibition of bacterial attachment to epithelial cells and subsequent inflammatory cytokine production. *J. Immunol.* **162**, 3559–3565.
32. Lavelle, E. C., McNeela, E., Armstrong, M. E., Leavy, O., Higgins, S. C., Mills, K. H. (2003) Cholera toxin promotes the induction of regulatory T cells specific for bystander antigens by modulating dendritic cell activation. *J. Immunol.* **171**, 2384–2392.
33. McGuirk, P., Mills, K. H. (2002) Pathogen-specific regulatory T cells provoke a shift in the Th1/Th2 paradigm in immunity to infectious diseases. *Trends Immunol.* **23**, 450–455.
34. Doetzel, A., Satoguina, J., Burchard, G., Rau, T., Loliger, C., Fleischer, B., Hoerauf, A. (2000) Antigen-specific cellular hyporesponsiveness in a chronic human helminth infection is mediated by T(h)3/T(r)1-type cytokines IL-10 and transforming growth factor- β but not by a T(h)1 to T(h)2 shift. *Int. Immunol.* **12**, 623–630.
35. Mendel, I., Shevach, E. M. (2002) The IL-10-producing competence of Th2 cells generated in vitro is IL-4 dependent. *Eur. J. Immunol.* **32**, 3216–3224.
36. Phipps, P. A., Stanford, M. R., Sun, J. B., Xiao, B. G., Holmgren, J., Shinnick, T., Hasan, A., Mizushima, Y., Lehner, T. (2003) Prevention of mucosally induced uveitis with a HSP60-derived peptide linked to cholera toxin B subunit. *Eur. J. Immunol.* **33**, 224–232.
37. Sun, J. B., Xiao, B. G., Lindblad, M., Li, B. L., Link, H., Czerkinsky, C., Holmgren, J. (2000) Oral administration of cholera toxin B subunit conjugated to myelin basic protein protects against experimental autoimmune encephalomyelitis by inducing transforming growth factor- β -secreting cells and suppressing chemokine expression. *Int. Immunol.* **12**, 1449–1457.
38. Thornton, A. M., Shevach, E. M. (1998) CD4⁺CD25⁺ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J. Exp. Med.* **188**, 287–296.
39. Walker, L. S., Chodos, A., Eggena, M., Dooks, H., Abbas, A. K. (2003) Antigen-dependent proliferation of CD4⁺ CD25⁺ regulatory T cells in vivo. *J. Exp. Med.* **198**, 249–258.
40. Yamazaki, S., Iyoda, T., Tarbell, K., Olson, K., Velinzon, K., Inaba, K., Steinman, R. M. (2003) Direct expansion of functional CD25⁺ CD4⁺ regulatory T cells by antigen-processing dendritic cells. *J. Exp. Med.* **198**, 235–247.
41. Jonuleit, H., Schmitt, E., Steinbrink, K., Enk, A. H. (2001) Dendritic cells as a tool to induce anergic and regulatory T cells. *Trends Immunol.* **22**, 394–400.
42. Rissoan, M. C., Soumelis, V., Kadowaki, N., Grouard, G., Briere, F., de Waal Malefyt, R., Liu, Y. J. (1999) Reciprocal control of T helper cell and dendritic cell differentiation. *Science* **283**, 1183–1186.
43. Boonstra, A., Asselin-Paturel, C., Gilliet, M., Crain, C., Trinchieri, G., Liu, Y. J., O'Garra, A. (2003) Flexibility of mouse classical and plasmacytoid-derived dendritic cells in directing T helper type 1 and 2 cell development: dependency on antigen dose and differential Toll-like receptor ligation. *J. Exp. Med.* **197**, 101–109.
44. de Jong, E. C., Vieira, P. L., Kalinski, P., Schuitemaker, J. H., Tanaka, Y., Wierenga, E. A., Yazdanbakhsh, M., Kapsenberg, M. L. (2002) Microbial compounds selectively induce Th1 cell-promoting or Th2 cell-promoting dendritic cells in vitro with diverse Th cell-polarizing signals. *J. Immunol.* **168**, 1704–1709.
45. Gagliardi, M. C., Sallusto, F., Marinaro, M., Langenkamp, A., Lanzavecchia, A., De Magistris, M. T. (2000) Cholera toxin induces maturation of human dendritic cells and licenses them for Th2 priming. *Eur. J. Immunol.* **30**, 2394–2403.
46. MacDonald, A. S., Straw, A. D., Dalton, N. M., Pearce, E. J. (2002) Cutting edge: Th2 response induction by dendritic cells: a role for CD40. *J. Immunol.* **168**, 537–540.
47. Lespagnard, L., Mettens, P., De Smedt, T., Bazin, H., Urbain, J., Leo, O., Moser, M. (1998) The immune response induced in vivo by dendritic cells is dependent on B7-1 or B7-2, but the inhibition of both signals does not lead to tolerance. *Int. Immunol.* **10**, 295–304.
48. Martin, E., O'Sullivan, B., Low, P., Thomas, R. (2003) Antigen-specific suppression of a primed immune response by dendritic cells mediated by regulatory T cells secreting interleukin-10. *Immunity* **18**, 155–167.
49. Fontana, S., Moratto, D., Mangal, S., De Francesco, M., Vermi, W., Ferrari, S., Facchetti, F., Kutukculer, N., Fiorini, C., Duse, M., Das, P. K., Notarangelo, L. D., Plebani, A., Badolato, R. (2003) Functional defects of dendritic cells in patients with CD40 deficiency. *Blood* **102**, 4099–4106.
50. O'Garra, A. (1998) Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity* **8**, 275–283.
51. Murphy, K. M., Reiner, S. L. (2002) The lineage decisions of helper T cells. *Nat. Rev. Immunol.* **2**, 933–944.
52. Iwasaki, A., Kelsall, B. L. (1999) Freshly isolated Peyer's patch, but not spleen, dendritic cells produce interleukin 10 and induce the differentiation of T helper type 2 cells. *J. Exp. Med.* **190**, 229–239.
53. Rincon, M., Anguita, J., Nakamura, T., Fikrig, E., Flavell, R. A. (1997) Interleukin (IL)-6 directs the differentiation of IL-4-producing CD4⁺ T cells. *J. Exp. Med.* **185**, 461–469.
54. Maldonado-Lopez, R., Maliszewski, C., Urbain, J., Moser, M. (2001) Cytokines regulate the capacity of CD8 α ⁺ and CD8 α ⁺ dendritic cells to prime Th1/Th2 cells in vivo. *J. Immunol.* **167**, 4345–4350.
55. Chen, Y., Kuchroo, V. K., Inobe, J., Hafler, D. A., Weiner, H. L. (1994) Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science* **265**, 1237–1240.
56. Seder, R. A., Marth, T., Sieve, M. C., Strober, W., Letterio, J. J., Roberts, A. B., Kelsall, B. (1998) Factors involved in the differentiation of TGF- β -producing cells from naive CD4⁺ T cells: IL-4 and IFN- γ have opposing effects, while TGF- β positively regulates its own production. *J. Immunol.* **160**, 5719–5728.
57. Cong, Y., Oliver, A. O., Elson, C. O. (2001) Effects of cholera toxin on macrophage production of co-stimulatory cytokines. *Eur. J. Immunol.* **31**, 64–71.
58. Diehl, S., Rincon, M. (2002) The two faces of IL-6 on Th1/Th2 differentiation. *Mol. Immunol.* **39**, 531–536.
59. Constant, S. L., Brogdon, J. L., Piggott, D. A., Herrick, C. A., Visintin, L., Ruddle, N. H., Bottomly, K. (2002) Resident lung antigen-presenting cells have the capacity to promote Th2 T cell differentiation in situ. *J. Clin. Invest.* **110**, 1441–1448.
60. Nakae, S., Komiyama, Y., Yokoyama, H., Nambu, A., Umeda, M., Iwase, M., Homma, I., Sudo, K., Horai, R., Asano, M., Iwakura, Y. (2003) IL-1 is required for allergen-specific Th2 cell activation and the development of airway hypersensitivity response. *Int. Immunol.* **15**, 483–490.
61. Braun, M. C., He, J., Wu, C. Y., Kelsall, B. L. (1999) Cholera toxin suppresses interleukin (IL)-12 production and IL-12 receptor β 1 and β 2 chain expression. *J. Exp. Med.* **189**, 541–552.
62. Burkart, V., Kim, Y. E., Hartmann, B., Ghiea, I., Sydath, U., Kauer, M., Fingberg, W., Hanifi-Moghaddam, P., Muller, S., Kolb, H. (2002) Cholera toxin B pretreatment of macrophages and monocytes diminishes their proinflammatory responsiveness to lipopolysaccharide. *J. Immunol.* **168**, 1730–1737.
63. Yura, M., Takahashi, I., Terawaki, S., Hiroi, T., Kweon, M. N., Yuki, Y., Kiyono, H. (2001) Nasal administration of cholera toxin (CT) suppresses clinical signs of experimental autoimmune encephalomyelitis (EAE). *Vaccine* **20**, 134–139.

64. Gaupp, S., Hartung, H. P., Toyka, K., Jung, S. (1997) Modulation of experimental autoimmune neuritis in Lewis rats by oral application of myelin antigens. *J. Neuroimmunol.* **79**, 129–137.
65. Tsuru, S., Taniguchi, M., Shinomiya, N., Fujisawa, H., Zinnaka, Y., Nomoto, K. (1987) Cholera toxin-induced tolerance to allografts in mice. *Immunology* **61**, 77–83.
66. Sun, J. B., Li, B. L., Czerkinsky, C., Holmgren, J. (2000) Enhanced immunological tolerance against allograft rejection by oral administration of allogeneic antigen linked to cholera toxin B subunit. *Clin. Immunol.* **97**, 130–139.
67. Sun, J. B., Rask, C., Olsson, T., Holmgren, J., Czerkinsky, C. (1996) Treatment of experimental autoimmune encephalomyelitis by feeding myelin basic protein conjugated to cholera toxin B subunit. *Proc. Natl. Acad. Sci. USA* **93**, 7196–7201.
68. Asford, C., Czerkinsky, C., Durand, A., Stefanutti, A., Thivolet, C. (2002) $\alpha 4$ Integrins and L-selectin differently orchestrate T-cell activity during diabetes prevention following oral administration of CTB-insulin. *J. Autoimmun.* **19**, 223–232.
69. Boirivant, M., Fuss, I. J., Ferroni, L., De Pascale, M., Strober, W. (2001) Oral administration of recombinant cholera toxin subunit B inhibits IL-12-mediated murine experimental (trinitrobenzene sulfonic acid) colitis. *J. Immunol.* **166**, 3522–3532.
70. Kim, N., Cheng, K. C., Kwon, S. S., Mora, R., Barbieri, M., Yoo, T. J. (2001) Oral administration of collagen conjugated with cholera toxin induces tolerance to type II collagen and suppresses chondritis in an animal model of autoimmune ear disease. *Ann. Otol. Rhinol. Laryngol.* **110**, 646–654.
71. Bregenholt, S., Wang, M., Wolfe, T., Hughes, A., Baerentzen, L., Dyrberg, T., von Herrath, M. G., Petersen, J. S. (2003) The cholera toxin B subunit is a mucosal adjuvant for oral tolerance induction in type I diabetes. *Scand. J. Immunol.* **57**, 432–438.
72. Luross, J. A., Heaton, T., Hirst, T. R., Day, M. J., Williams, N. A. (2002) *Escherichia coli* heat-labile enterotoxin B subunit prevents autoimmune arthritis through induction of regulatory CD4⁺ T cells. *Arthritis Rheum.* **46**, 1671–1682.