Published in final edited form as:
Immunol Invest. 2010 ; 39(4-5): 449–467.

Heat-labile Enterotoxins as Adjuvants or Anti-Inflammatory Agents

Shuang Liang[1] and **George Hajishengallis**[1,2,*]

[1] University of Louisville School of Dentistry, Oral Health and Systemic Disease, Louisville, KY, USA

[2] University of Louisville School of Medicine, Department of Microbiology and Immunology, Louisville, KY, USA

Abstract

Escherichia coli and *Vibrio cholerae* produce structurally related AB_5-type heat-labile enterotoxins which are classified into two major types. The Type I subfamily includes cholera toxin and *E. coli* LT-I, whereas the Type II subfamily comprises LT-IIa and LT-IIb. In addition to their roles in microbial pathogenesis, the enterotoxins are widely and intensively studied for their exceptionally strong adjuvant and immunomodulatory activities, which are not necessarily dependent upon their abilities to elevate intracellular cAMP levels. Despite general structural similarities, these molecules, in intact or derivative form, display notable differences in their interactions with gangliosides or Toll-like receptors. This divergence results in differential immune response outcomes, the underlying mechanisms of which remain largely uncharacterized. Whereas the study of these molecules has been pivotal in understanding basic mechanisms of immune regulation, a formidable challenge is to dissociate toxicity from useful properties that can be exploited in vaccine development or for the treatment of autoimmune inflammatory diseases.

Introduction

The heat-labile enterotoxins of *Escherichia coli* and *Vibrio cholerae* have been extensively studied for their virulence in microbial infections and for their immunomodulatory properties (Connell 2007; Hajishengallis et al. 2005a; Holmes et al. 1995; Lavelle et al. 2004). These toxins are classified into two major types on the basis of genetic, biochemical, and immunological properties (Holmes et al. 1995). Type I includes the cholera toxin (CT) and the *E. coli* heat-labile enterotoxin (LT-I), whereas Type II includes *E. coli* LT-IIa and LT-IIb. Both types share an AB_5 oligomeric structure in which an enzymatically active and toxic A subunit is noncovalently linked to a pentameric ganglioside-binding (B_5) subunit (Gill et al. 1981; van den Akker et al. 1996). The main antigenic differences between Type I and Type II enterotoxins are due to significant divergence in the amino-acid sequence of their B subunits, which share less than 14% amino-acid sequence identity, resulting in differential binding to ganglioside receptors (Holmes et al. 1995). Type I toxins bind with high affinity to GM1 ganglioside. On the other hand, LT-IIa displays a more promiscuous binding profile, which includes GD1b, GD1a, and GM1, in order of decreasing affinity, whereas LT-IIb, the B subunit of which shares 56% aminoacid sequence identity with that of LT-IIa, lacks affinity for GM1 or GD1b but binds avidly to GD1a (Holmes et al. 1995).

*To whom correspondence should be addressed. 501 South Preston St., Room 206, Louisville, KY 40202, Tel. 502-852-5276, g0haji01@louisville.edu.

The actual catalytic moiety of the A subunit is the A1 subcomponent, which is formed by proteolytic cleavage and reduction of an intrachain disulfide bond, while the *C*-terminal A2 subcomponent is inserted noncovalently into the central pore of the ring-shaped B pentamer (Gill et al. 1981; van den Akker et al. 1996). The B subunit is nontoxic by itself but, upon high-affinity binding to cell surface gangliosides, delivers the A subunit intracellularly. The internalized A subunit can then activate the Gsα component of adenylate cyclase via its ADP-ribosyltransferase activity, leading to unregulated elevation of intracellular cAMP (Holmes et al. 1995). In gut epithelial cells, cAMP elevation leads to massive secretion of electrolytes and water into the gut lumen, which is clinically manifested as diarrhea in humans and animals. As a consequence of this intrinsic toxicity, the use of intact enterotoxins as adjuvants in human vaccine formulations is precluded, despite their potent mucosal adjuvanticity in experimental animal immunizations (Hajishengallis et al. 2005a). The paucity of adjuvants licensed for human use, however, and the tremendous health impact of infectious diseases, which remain leading causes of mortality and morbidity, create an urgent need to develop novel and improved adjuvants (Holmgren and Czerkinsky 2005; Kwissa et al. 2007; Mestecky et al. 2008). In this regard, research on the adjuvant properties of enterotoxins is performed under the premise that it is possible to dissociate useful adjuvant properties from undesirable toxic effects.

In addition to its toxic effects on gut epithelial cells, elevated intracellular cAMP mediates important regulatory effects in a variety of immune cell types, mainly through activation of protein kinase A (PKA) and downstream phosphorylation of additional kinases or transcription factors that act through cAMP-responsive elements in the promoter region of target genes (Lalli and Sassone-Corsi 1994). In this context, the enterotoxins exert a variety of regulatory effects that could be harnessed for the treatment of autoimmune inflammatory diseases, although some of these effects can be exerted in the absence of cAMP signaling (Gaupp et al. 1997; Sun et al. 1996; Yura et al. 2001). This review will summarize and discuss cAMP-dependent and –independent properties of heat-labile enterotoxins and how these impact on immunity and inflammation.

Immunostimulatory properties of enterotoxins: Role of cAMP and ganglioside binding

Most infectious diseases occur, or are initiated, at mucosal surfaces. Indeed, the vast majority of pathogens colonize or invade the mucosae via oral, respiratory, or urogenital routes (Holmgren and Svennerholm 2005; Mestecky et al. 2008). At least in principle, oral and other mucosal infectious diseases could be prevented by mucosal immunization for induction of specific secretory IgA antibodies (Hajishengallis and Russell 2008; Holmgren and Czerkinsky 2005) which can interfere with microbial adherence and colonization (Hajishengallis et al. 1992). Purified vaccine proteins, however, generally do not stimulate immune responses, at least in part, due to failure to induce appropriate activation signals in antigen-presenting cells (APC) (Akira et al. 2001; Hajishengallis et al. 2005a; Kwissa et al. 2007). In fact, mucosal administration of protein immunogens without adjuvants often induces a state of immunologic unresponsiveness (tolerance) rather than active immunity (Czerkinsky et al. 1999). APC activation signals, nevertheless, are readily induced by noxious microbial molecules, or intact pathogens, and are essential for bridging innate immune recognition to activation of adaptive immunity (Akira et al. 2001; Iwasaki and Medzhitov 2004). Induction of such activating signals is a major mechanism of action for adjuvants, although their immunostimulatory effects should be free of concomitant toxicity.

In their efforts to identify adjuvants that could overcome tolerance and induce active immune responses, researchers have discovered that heat-labile enterotoxins possess exceptionally potent mucosal adjuvant properties and act through activation of a variety of

immunocompetent cells (Elson and Dertzbaugh 2005). Early work on the adjuvanticity of enterotoxins was performed using CT or LT-I. In terms of mechanisms of action, CT was shown to enhance the antigen-presenting capacity of APC by upregulating the expression of co-stimulatory molecules and MHC class II (Bromander et al. 1991; Cong et al. 1997). Moreover, CT promotes B cell isotype-switch differentiation and stimulates production of IgA and IgG antibodies (Holmgren et al. 1993). Although CT displays complex stimulatory or inhibitory effects on T cell proliferation in vitro (Elson et al. 1995), it is well established that it promotes Th2 responses in vivo, partly through induction of IL-4 (Marinaro et al. 1995; Munoz et al. 1990). Moreover, CT proactively inhibits Th1 responses by inhibiting APC induction of IL-12 through cAMP-dependent suppression of the interferon regulatory factor-8, a critical transcription factor for IL-12 p35 and p40 gene expression (la Sala et al. 2009). Another mechanism whereby CT can inhibit IL-12 induction is through inhibition of CD40 ligand (CD40L) expression on CD4+ T cells (Martin et al. 2001). This results in suppression of CD40-CD40L interactions between APC and T cells which would otherwise promote the production of IL-12 other (Martin et al. 2001). Interruption of CD40-CD40L interactions between B cells and T helper cells can moreover stop B cell proliferation and initiate their differentiation into plasma cells (Liu and Banchereau 1997).

LT-I shares at least some of the immunostimulatory properties of CT, as it can also stimulate antigen presentation via upregulation of costimulatory molecules and MHC Class II expression (Arce et al. 2005; Nashar et al. 1997). Furthermore, LT-I can similarly interfere with CD40-CD40L interactions between APC and T cells through its ability to downregulate CD40 expression on dendritic cells (Petrovska et al. 2003). However, LT-I appears to promote a more balanced T cell response involving aspects of both Th1 and Th2 responses (Takahashi et al. 1996).

Observations that pure B pentamers (CT-B or LT-I-B) are relatively weak adjuvants and that a point mutant (E112K) of LT-I, which lacks ADP-ribosylating activity, is essentially devoid of adjuvant properties (Lycke et al. 1992), initially suggested a link between enzymatic activity and adjuvanticity. That the ADP-ribosylating activity of the A1 subunit plays a role in adjuvanticity is additionally suggested by an adjuvant construct, designated CTA1-DD. In this construct, the toxic A1 subunit of CT is coupled to the Ig-binding domain of staphylococcal protein A and is thus targeted to B cells in a GM1-independent manner (Agren et al. 1999). Although the adjuvanticity of CTA1-DD is dependent upon intact enzymatic activity, the construct is devoid of toxicity, raising the possibility for cAMP-independent but still enzymatically dependent adjuvanticity. Moreover, despite the original results with the E112K mutant of LT-I (Lycke et al. 1992), subsequent work showed that a number of other catalytically defective mutants of LT-I or CT retain significant adjuvanticity, although the mechanisms of action remain largely uncharacterized (Fontana et al. 1995; Pizza et al. 1994; Pizza et al. 2001; Yamamoto et al. 1999). The results of these studies imply that neither induction of cAMP nor enzymatic activity is essential for adjuvant action. Interestingly, elevation of intracellular cAMP is not even sufficient for adjuvanticity since forskolin, a potent adenylate cyclase activator, lacks adjuvant activity (Wilson et al. 1993).

Furthermore, if the cAMP-inducing activity of the enterotoxins was a crucial and determining factor for adjuvanticity, one would not expect significant differences in adjuvant mechanisms between the Type I and the equipotent (in terms of ADP-ribosylating activity) Type II enterotoxins. In fact, this is not the case since CT, LT-IIa, and LT-IIb induce distinct type of mucosal immune responses to the same co-administered immunogen (Arce et al. 2005; Martin et al. 2000). Moreover, in contrast to Type I enterotoxins, LT-IIa and LT-IIb do not influence CD40L expression on $CD4^+$ T cells (Martin et al. 2001) and are thus biased to skew immune responses more towards Th1, as seen in vivo especially for LT-

IIb (Martin et al. 2000) (Table 1). Another possible consequence of the failure to interrupt CD40-CD40L signaling could be that Type II enterotoxins may preferentially foster the formation of memory B cells, a process that depends upon the continuation of CD40-CD40L interactions (Liu and Banchereau 1997). These differences suggest that it is possibly the different ganglioside-binding specificities of these enterotoxins that determine distinct immunoregulatory effects. In this regard, CT, LT-IIa, and LT-IIb bind differentially to immunocompetent cells, as a result of their different ganglioside-binding specificities (Arce et al. 2005; Martin et al. 2000). Additional mechanistic insights, regarding the roles of gangliosides in adjuvanticity and tocxicity, have been obtained using modified versions of LT-IIa and LT-IIb with intact A subunits but mutated B subunits (LT-IIa[T34I] and LT-IIb[T13I]). As expected, these point mutations resulted in altered ganglioside-binding activities, but, intriguingly, the constructs lost cAMP-inducing activity and retained significant adjuvanticity (Nawar et al. 2005). These findings suggest that toxicity and adjuvanticity may be mediated by different gangliosides, although other, unidentified receptors cannot be excluded. If the ganglioside specificities are important determining factors of enterotoxin immunomodulatory properties, one would also expect to find differences between LT-IIa and LT-IIb. Indeed, LT-IIa (and CT), but not LT-IIb, selectively induces apoptosis in $CD8^+$ T cells (Arce et al. 2005; Elson and Dertzbaugh 2005; Nashar et al. 1996) (Table 1), implying a role for GM1 binding in this function. Since $CD8^+$ cells constitute a major source of IFN-γ, which promotes cell-mediated immunity and fosters the development of Th1 cells (Sad et al. 1995), this may explain the LT-IIb bias for supporting Th1 responses, although additional unidentified mechanisms are likely involved. In summary, Type I and Type II holotoxins arguably contain A subunit-dependent adjuvanticity that is independent of their cAMP-inducing activity, whereas their ganglioside-binding characteristics determine in large part the nature of their immunomodulatory effects.

Non-ganglioside receptors and enterotoxin adjuvanticity: Interactions with TLRs

Although gangliosides are prominent receptors for Type I or Type II enterotoxins, it is becoming increasingly evident that at least some of the immunomodulatory properties of the enterotoxins may involve interactions with additional receptors. In this regard, point mutations in the B subunits of CT (H57A) or LT-I (H57S) render the molecules defective in immunomodulatory signaling and toxicity despite retaining high-affinity binding to GM1 ganglioside (Aman et al. 2001). It is thus conceivable that structural alterations in these mutants, while not preventing binding to GM1, may preclude interactions with additional, cooperative receptor(s) required for signaling. Furthermore, as alluded to above, the participation of non-ganglioside receptors in mediating the adjuvant effects of LT-IIa(T34I) and LT-IIb(T13I) (Nawar et al. 2005) cannot be formally ruled out. In this context, microbial ligand interactions with a single receptor often represent an oversimplified model. Rather, cellular activation by microbial molecules may actually involve interactions with several co-operating host receptors within lipid rafts, which function as cellular signaling platforms (Simons and Toomre 2000; Triantafilou et al. 2002).

In our efforts to identify immunostimulatory activities mediated exclusively by the non-catalytic B pentameric subunits, we discovered that a member of the Toll-like receptor (TLR) family of pattern-recognition receptors (Beutler et al. 2006), the TLR2, is uniquely activated by the B pentamers of Type II but not Type I enterotoxins (Hajishengallis et al. 2005b). This finding suggested a novel mechanism whereby enterotoxin derivatives can be exploited as vaccine adjuvants, *i.e*., by eliciting TLR-dependent immunostimulatory activity. In this context, TLRs, by virtue of their ability to detect and respond to conserved structures from pathogens (*e.g*., lipopolysaccharide (LPS) and lipopeptides), play a key role in stimulating APC function and bridging innate to adaptive immunity (Akira et al. 2001;

Iwasaki and Medzhitov 2004). To harness these TLR properties, TLR agonists and synthetic analogues are currently major targets for developing vaccine adjuvants to prevent infectious diseases (Gearing 2007).

Mechanistic investigation of the B pentamer of LT-IIb (LT-IIb-B_5) has shown that its interaction with TLR2 takes place in lipid rafts and is actually facilitated by the lipid raft-resident GD1a ganglioside (Liang et al. 2007a) (Fig. 1). Moreover, binding to TLR2 is mediated by the hydrophobic upper pore region of LT-IIb-B_5 (Liang et al. 2009a). TLR1 serves as a signaling partner of TLR2 and is similarly recruited to lipid rafts, whereas the intracellular adaptor proteins TIRAP and MyD88 colocalize with the LT-IIb-B_5 receptor complex (GD1a/TLR2/TLR1) and are essential for activation of nuclear factor (NF)-κB and induction of cytokines by LT-IIb-B_5 (Liang et al. 2007a; Liang et al. 2009b) (Fig. 1). As a consequence of its TLR2/TLR1-activating capacity, LT-IIb-B_5 provides appropriate APC-activating signals, manifested as upregulated expression of costimulatory molecules (CD40, CD54, CD80, CD86) and MHC Class II, and production of TNF-α and IL-6. Moreover, LT-IIb-B_5 induces functional costimulation of dendritic cells, which thereby stimulate $CD4^+$ T cell proliferation and production of immunostimulatory cytokines. These adjuvant effects are dependent on TLR2 and its signaling adaptor MyD88 (Liang et al. 2009a; Liang et al. 2009b). Moreover, in cocultures of LT-IIb-B_5-stimulated dendritic cells and $CD4^+$ T cells, the relative numbers of T regulatory cells (Tregs) are decreased, whereas the numbers of Th1, Th17, and especially Th2 are increased (Liang and Hajishengallis; unpublished observations), in contrast to the intact holotoxin, which preferentially induces Th1 (Martin et al. 2000). Therefore, it appears that LT-IIb-B_5-activated dendritic cells cause an in imbalance between Treg and other helper T cell subsets, favoring the expansion of T cell effector subsets. In fact, it is possible that LT-IIb-B_5 may be able to directly suppress the activity of Tregs. This is because TLR2 signaling transiently suppresses the induction of the transcription factor Foxp3 in Tregs, which are thereby temporarily switched off and allow strong activation of effector T cells (Liu et al. 2006). These in vitro properties readily explain why LT-IIb-B_5 functions as a useful in vivo adjuvant (Liang et al. 2009b). Importantly, this B pentamer stimulates induction of salivary IgA response to a mucosally co-administered immunogen at levels comparable to that achieved using intact LT-IIb adjuvant. However, the holotoxin is more potent in augmenting vaginal IgA or serum IgG antibody responses (Liang et al. 2009b). Similarly to LT-IIb-B_5, the related B pentamer of the LT-IIa enterotoxin (LT-IIa-B_5) also induces TLR2-dependent activation of antigen-presenting function resulting in enhanced $CD4^+$ T cell proliferation and augmentation of mucosal antibody responses in vivo (Terry Connell; personal communication).

In contrast to its B pentamer, intact LT-IIb does not bind or activate TLR2, owing to the presence of the A subunit which sterically interferes with TLR2 binding (Liang et al. 2007b). In this regard, the TLR2-binding site of LT-IIb-B_5 is defined by a ring of four upper region residues (M69, A70, L73, and S74) (Liang et al. 2009a), which, strikingly, are also critical for hydrophobic interactions between the B pentamer and the A2 segment of the A subunit in the fully-assembled LT-IIb holotoxin (van den Akker et al. 1996). As a result, the TLR2-binding site is blocked in the intact holotoxin. Intriguingly, M69, A70, L73, and S74 are exactly shared by LT-IIa-B_5, the only other known enterotoxin B pentamer that activates TLR2 (Hajishengallis et al. 2005b), but not by the Type I B pentamers, LT-I-B_5 and CT-B_5 (van den Akker et al. 1996).

Anti-inflammatory effects of enterotoxins and applications for suppressing autoimmunity

Both Type I and Type II enterotoxins (CT, LT-IIa and LT-IIb) inhibit NF-κB activation and production of TNF-α and IL-8 in monocytic cells stimulated with LPS or other

proinflammatory stimuli (Hajishengallis et al. 2004). However, their respective B pentamers lack this regulatory activity, suggesting possible involvement of cAMP signaling (Hajishengallis et al. 2004). Indeed, membrane-permeable cAMP analogs and cAMP-elevating agonists (*e.g.*, forskolin) mimick the anti-inflammatory action of LT-IIb, whereas pharmacological inhibition of cAMP synthesis, or inhibition of its downstream target PKA, completely reverses the inhibitory effect of LT-IIb on cell activation (Liang et al. 2007b). Furthermore, catalytically-defective point mutants of LT-IIb, which do not elevate cAMP, fail to inhibit NF-κB activation and proinflammatory cytokine induction (Liang et al. 2007b).

CT has been studied more extensively than Type II toxins for its anti-inflammatory properties and was shown to additionally inhibit induction of IL-12, macrophage-inflammatory protein (MIP)-1α, MIP-1β, and monocyte chemoattractant protein-1 (MCP-1) in dendritic cells (reviewed by Lavelle et al. 2004). On the other hand, CT can induce the production of certain other cytokines, like IL-6 and IL-10, the transcription of which is minimally dependent upon or independent of NF-κB activation. For instance, the genes for IL-6 and IL-10 contain cAMP-responsive elements and their transcription is positively regulated by cAMP (Brenner et al. 2003; Krueger et al. 1991).

Although Type II enterotoxins induce little or no proinflammatory cytokine release, their respective B pentamers (LT-IIa-B_5 and especially LT-IIb-B_5) induce high levels of proinflammatory cytokines in human or mouse monocytes/macrophages, attributable to their TLR2-stimulating activity (Hajishengallis et al. 2004; Hajishengallis et al. 2005b). Moreover, the ability of the Type II B pentamers to induce NF-κB activation is antagonized by the holotoxins, as long as they are catalytically active (Hajishengallis et al. 2004; Liang et al. 2007b) (Fig. 2). This inhibitory effect is minimally dependent on endogenous production of IL-10, even though the combination of holotoxins and B pentamers synergistically induces IL-10 (Hajishengallis et al. 2004). Rather, the antagonistic effect of the holotoxins is mediated through their ability to activate cAMP-dependent PKA signaling (Liang et al. 2007b) (Fig. 2). In this regard, PKA has been shown to phosphorylate the cAMP response element-binding protein (CREB), which can thereby effectively compete with the p65 subunit of NF-κB for limiting amounts of a common transcriptional co-activator, the CREB-binding protein (CBP) (Parry and Mackman 1997). Consequently, this leads to decreased NF-κB activation and reduced transcription of NF-κB-dependent cytokine genes like TNF-α. In addition, PKA was also shown to phosphorylate glycogen synthase kinase-3β on Ser9 (Fang et al. 2000), an event that inactivates this kinase that would otherwise positively regulate cell activation (Martin et al. 2005).

It is conceivable that the ability of Type I or Type II II enterotoxins to inhibit LPS-induced cellular activation and to suppress the inherent proinflammatory potential of their own B subunits (Hajishengallis et al. 2004; Liang et al. 2007b) may serve to downregulate innate immunity and increase the survival capacity of enterotoxigenic *E. coli* or *Vibrio cholerae*, thereby prolonging infections with these pathogens. Whereas this concept has not been experimentally addressed, the in vitro anti-inflammatory behavior of the enterotoxins is consistent with in vivo observations. Specifically, cholera toxin is non-inflammatory in an animal model at doses that readily induce intestinal fluid secretion (Triadafilopoulos et al. 1989). This is in stark contrast to the proinflammatory *Clostridium difficile* toxin A, another toxin which causes intestinal fluid secretion (Triadafilopoulos et al. 1989). Moreover, in humans, *V. cholerae* induces either non-inflammatory diarrhea or inflammatory gastroenteritis, determined exclusively by a single virulence factor, cholera toxin. Specifically, cholera toxin-producing strains induce watery diarrhea in the absence of inflammation, whereas strains that do not produce cholera toxin cause inflammatory diarrhea (Satchell 2003). Similar observations have not yet been made for enterotoxigenic *E.*

coli, although it is reasonable to assume that the presence of LT-I or LT-II toxins may downregulate inflammatory responses.

As mentioned above, the same four residues of LT-IIb-B_5 residues that mediate TLR2 binding are also critical for hydrophobic interactions with the A2 segment in the fully-assembled LT-IIb (van den Akker et al. 1996; Liang et al. 2009a) and, therefore, the TLR2-binding site is masked in the intact holotoxin (Liang et al. 2007b). Since evolution does not work with preconceived plans to generate a fully assembled AB_5 toxin by design, the expression of the B pentamer in free form is likely to predate the fully assembled LT-IIb molecule. Despite the lack of any relevant data, it is intriguing to speculate that, at least in part, the evolutionary pressure for the development of fully assembled LT-IIb holotoxin was to suppress TLR2-induced activation of innate immunity in response to the free B pentamer.

Tregs constitute an important subset of helper T cells capable of suppressing the activation of effector T cells. An additional mechanism whereby CT can exert anti-inflammatory action is through induction of antigen-specific Tregs which secrete high levels of IL-10 and IL-13 but little or no IL-4 (Lavelle et al. 2003). This is consistent with findings that cAMP induces IL-10 and IL-13 but not IL-4 in Th2 cells (Chen et al. 2000), which may give rise to Tregs if they lose the ability to produce IL-4 (Mendel and Shevach 2002). As a result of its strong anti-inflammatory action, CT has been successfully used to suppress T cell-mediated autoimmune conditions, such as experimental autoimmune encephalomyelitis (Yura et al. 2001) and experimental autoimmune neuritis (Gaupp et al. 1997), models for multiple sclerosis and inflammatory demyelinating neuropathies, respectively.

However, protection against experimental autoimmune encephalomyelitis was also observed after oral administration of CT-B linked to myelin basic protein (Sun et al. 1996). Similarly, oral delivery of CT-B conjugated to a peptide from the heat-shock protein-60 (Phipps et al. 2003) or to insulin (Aspord et al. 2002) inhibits induction of experimental uveitis or diabetes, respectively. Although the immunosuppressive mechanism of orally administered CT-B is cAMP-independent, it appears to involve induction of TGF-β at the effector sites (Phipps et al. 2003; Sun et al. 2000). The B pentamers from LT-IIa and LT-IIb have not been investigated for therapeutic tolerance induction; however, both molecules are unlikely to have such function given their ability for TLR2-mediated cell activation (Hajishengallis et al. 2005b; Liang et al. 2009a). On the other hand, like CT-B, LT-I-B has been exploited for induction of mucosal tolerance against autoimmune inflammatory conditions, such as collagen-induced arthritis (Luross et al. 2002). Since LT-I-B inhibits Th1 development without concomitant activation of Th2, it was originally thought that its protective effect against collagen-induced arthritis involved its Th1-suppressive activity. Recent evidence, however, has implicated Th17, a novel Th cell subset characterized by IL-17 production, rather than the Th1 lineage in the development of collagen-induced arthritis or experimental autoimmune encephalomyelitis (Weaver et al. 2007). The initial implication of Th1 was based on the apparent resistance of IL-12-deficient mice against these autoimmune diseases. However, these IL-12-deficient mice lacked the IL-12p40 subunit (as opposed to the unique IL-12p35 subunit), which is shared by IL-23, a heterodimer consisting of IL-12p40 and a unique IL-23p19 subunit (Trinchieri 2003). The importance of IL-23 in Th17 development and demonstrations that IL-23p19-deficient mice, but not IL-12p35-deficient mice, are resistant to collagen-induced arthritis and experimental autoimmune encephalomyelitis, has conclusively implicated Th17 and “acquitted” Th1 (Langrish et al. 2005; Murphy et al. 2003). It is, therefore, possible that LT-I-B might have inhibited Th17 in that model of collagen-induced arthritis (Luross et al. 2002). However, LT-I-B treatment has failed to inhibit Th17 and, in fact, promoted the proportion and number of Th17 cells, at least in a model of experimental autoimmune uveoretinitis (Raveney et al. 2008).

A dramatic Th17-drving effect was recently demonstrated for intranasally administered CT in mice (Lee et al. 2009). This effect was dependent on IL-6 induction, since the CT-induced Th17 activity was abrogated in IL-6-deficient mice (Lee et al. 2009). Since Th17 have been shown to mediate protection against a variety of infectious diseases, involving bacterial, fungal, or parasitic pathogens (Kolls and Linden 2004), this might be another mechanism whereby CT could be useful as a vaccine adjuvant, although, as stated above, any protective mechanism needs to be dissociated from toxicity. At a first sight, the Th17-inducing effect of CT appears to be inconsistent with its ability to promote Treg function. However, TGF-β is required for induction of both Th17 and T reg. Moreover, TGF-β-induced signals in the absence of signal transducer and activator of transcription protein-3 (STAT3), which is inducible by IL-6, drives the development of the Treg lineage (Gaffen and Hajishengallis 2008). Thus, IL-6 may be a crucial factor which determines whether CT can function as an adjuvant that promotes Th17 responses, or as an immunosuppressive agent that promotes Treg function.

Conclusions and future utility

The heat-labile enterotoxins are fascinating molecules in terms of their diverse, often complex, effects on the immune system and are ideal molecular tools for discovering basic mechanisms of adjuvanticity or immune regulation. However, from a translational point of view, the enterotoxins raise serious safety issues. Until recently, the adjuvant effect of heat-labile enterotoxins has been thought to depend on their ganglioside-binding and toxic-enzyme activities, although arguably certain catalytically defective mutants retain significant adjuvanticity. By contrast, the B subunits of type II enterotoxins are immunostimulatory by novel mechanisms that depend on TLR activation (Hajishengallis et al. 2005b; Liang et al. 2007a; Liang et al. 2007b; Liang et al. 2009a; Liang et al. 2009b). As a result, it was recently shown that Type II B pentamers display adjuvant properties, free of toxic effects associated with the use of intact holotoxins (Liang et al. 2009b). However, proof-of-concept studies for the utility of Type II B pentamers as adjuvants in stimulating protective immunity against mucosal pathogens are not yet available. Such preclinical studies in appropriate animal models are necessary, as are Phase I (safety) clinical trials, before these molecules are fully considered for practical applications in vaccines for human use. It should be noted, however, that native or recombinant CT-B, a Type I B pentamer, has an excellent safety record as a component of a licensed oral vaccine against cholera (van Loon et al., 1996). However, whether B pentamers, in general, or engineered holotoxin adjuvants lacking enterotoxicity are safe for applications in human vaccines can only be determined empirically.

Intriguingly, the mechanisms of adjuvant action of Type II B pentamers are antagonized by the intact holotoxins (Liang et al. 2007b), which are also potent, if not stronger, adjuvants. This, however, is only an apparent paradox since TLR activation is not an obligatory mechanism of adjuvant action. Indeed, unlike TLR-based adjuvants, the intact enterotoxins can elicit adjuvant activity in the absence of proinflammatory activity (Lavelle et al. 2003; Lavelle et al. 2004; Ryan et al. 2000; Satchell 2003). For instance, CT, the most widely studied enterotoxin, promotes the induction of Th2 cells and Tregs, whereas it proactively inhibits Th1 cell differentiation and induction of proinflammatory cytokines, such as IL-12 and TNF-α (Holmgren et al. 2003; la Sala et al. 2009; Lavelle et al. 2003; Lavelle et al. 2004; Satchell 2003) (Table 1). Nevertheless, it exhibits exceptionally potent adjuvant properties (Elson and Dertzbaugh 2005), although its anti-inflammatory action and ability to promote Treg function has also been exploited for the treatment of experimental autoimmune diseases (Gaupp et al. 1997; Yura et al. 2001). It appears unlikely that every useful property of Type I or Type II enterotoxins can be dissociated from toxicity. However, available data indicate that it is feasible to construct enterotoxin derivatives, with minimal or

no toxic effects, that can be used either for enhancing immunity or mitigating inflammatory tissue damage, although their safety and efficacy in preclinical models will need to be confirmed in clinical trials.

Acknowledgments

Studies performed in the authors' lab and cited in this review were supported by U.S. Public Health Service Grants DE015254 and DE017138 to GH.

References

Agren LC, Ekman L, Lowenadler B, Nedrud JG, Lycke NY. Adjuvanticity of the cholera toxin A1-based gene fusion protein, CTA1-DD, is critically dependent on the ADP-ribosyltransferase and Ig-binding activity. J Immunol. 1999; 162:2432–2440. [PubMed: 9973526]

Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol. 2001; 2:675–680. [PubMed: 11477402]

Aman AT, Fraser S, Merritt EA, Rodigherio C, Kenny M, Ahn M, Hol WGJ, Williams NA, Lencer WI, Hirst TR. A mutant cholera toxin B subunit that binds GM1- ganglioside but lacks immunomodulatory or toxic activity. Proc Natl Acad Sci USA. 2001; 98:8536–8541. [PubMed: 11447291]

Arce S, Nawar HF, Russell MW, Connell TD. Differential binding of *Escherichia coli* enterotoxins LT-IIa and LT-IIb and of cholera toxin elicits differences in apoptosis, proliferation, and activation of lymphoid cells. Infect Immun. 2005; 73:2718–2727. [PubMed: 15845474]

Aspord C, Czerkinsky C, Durand A, Stefanutti A, Thivolet C. α4 Integrins and L-selectin differently orchestrate T-cell activity during diabetes prevention following oral administration of CTB-insulin. J Autoimmun. 2002; 19:223–232. [PubMed: 12473243]

Beutler B, Jiang Z, Georgel P, Crozat K, Croker B, Rutschmann S, Du X, Hoebe K. Genetic analysis of host resistance: Toll-like receptor signaling and immunity at large. Annu Rev Immunol. 2006; 24:353–389. [PubMed: 16551253]

Brenner S, Prosch S, Schenke-Layland K, Riese U, Gausmann U, Platzer C. cAMP-induced Interleukin-10 promoter activation depends on CCAAT/enhancer-binding protein expression and monocytic differentiation. J Biol Chem. 2003; 278:5597–5604. [PubMed: 12493739]

Bromander A, Holmgren J, Lycke N. Cholera toxin stimulates IL-1 production and enhances antigen presentation by macrophages in vitro. J Immunol. 1991; 146:2908–2914. [PubMed: 1901890]

Chen CH, Zhang DH, LaPorte JM, Ray A. Cyclic AMP activates p38 mitogen-activated protein kinase in Th2 Cells: Phosphorylation of GATA-3 and stimulation of Th2 cytokine gene expression. J Immunol. 2000; 165:5597–5605. [PubMed: 11067915]

Cong Y, Weaver CT, Elson CO. The mucosal adjuvanticity of cholera toxin involves enhancement of costimulatory activity by selective up-regulation of B7.2 expression. J Immunol. 1997; 159:5301–5308. [PubMed: 9548469]

Connell TD. Cholera toxin, LT-I, LT-IIa and LT-IIb: the critical role of ganglioside binding in immunomodulation by type I and type II heat-labile enterotoxins. Expert Rev Vaccines. 2007; 6:821–834. [PubMed: 17931161]

Czerkinsky C, Anjuere F, McGhee JR, George-Chandy A, Holmgren J, Kieny MP, Fujiyashi K, Mestecky JF, Pierrefite-Carle V, Rask C, Sun JB. Mucosal immunity and tolerance: relevance to vaccine development. Immunol Rev. 1999; 170:197–222. [PubMed: 10566152]

Elson CO, Holland SP, Dertzbagh MT, Cuff CF, Anderson AO. Morphologic and functional alterations of mucosal T cells by cholera toxin and its B subunit. J Immunol. 1995; 154:1032–1040. [PubMed: 7822780]

Elson, CO.; Dertzbaugh, MT. Mucosal adjuvants. In: Mestecky, J.; Bienenstock, J.; Lamm, ME., et al., editors. Mucosal Immunology. San Diego: Elsevier/Academic Press; 2005. p. 967-986.

Fang X, Yu SX, Lu Y, Bast RC Jr, Woodgett JR, Mills GB. Phosphorylation and inactivation of glycogen synthase kinase 3 by protein kinase A. Proc Natl Acad Sci U S A. 2000; 97:11960–11965. [PubMed: 11035810]

Fontana MR, Manetti R, Giannelli V, Magagnoli C, Marchini A, Olivieri R, Domenighini M, Rappuoli R, Pizza M. Construction of nontoxic derivatives of cholera toxin and characterization of the immunological response against the A subunit. Infect Immun. 1995; 63:2356–2360. [PubMed: 7768621]

Gaffen SL, Hajishengallis G. A new inflammatory cytokine on the block: rethinking periodontal disease and the Th1/Th2 paradigm in the context of Th17 cells and IL-17. J Dent Res. 2008; 87:817–828. [PubMed: 18719207]

Gaupp S, Hartung HP, Toyka K, Jung S. Modulation of experimental autoimmune neuritis in Lewis rats by oral application of myelin antigens. Journal of Neuroimmunology. 1997; 79:129–137. [PubMed: 9394785]

Gearing AJH. Targeting toll-like receptors for drug development: a summary of commercial approaches. Immunol Cell Biol. 2007; 85:490–494. [PubMed: 17667933]

Gill DM, Clements JD, Robertson DC, Finkelstein RA. Subunit number and arrangement in *Escherichia coli* heat-labile enterotoxin. Infect Immun. 1981; 33:677–682. [PubMed: 7026442]

Hajishengallis G, Nikolova E, Russell MW. Inhibition of *Streptococcus mutans* adherence to saliva-coated hydroxyapatite by human secretory immunoglobulin A antibodies to the cell surface protein antigen I/II: Reversal by IgA1 protease cleavage. Infect Immun. 1992; 60:5057–5064. [PubMed: 1333448]

Hajishengallis G, Nawar H, Tapping RI, Russell MW, Connell TD. The Type II heat-labile enterotoxins LT-IIa and LT-IIb and their respective B pentamers differentially induce and regulate cytokine production in human monocytic cells. Infect Immun. 2004; 72:6351–6358. [PubMed: 15501764]

Hajishengallis G, Arce S, Gockel CM, Connell TD, Russell MW. Immunomodulation with enterotoxins for the generation of secretory immunity or tolerance: applications for oral infections. J Dent Res. 2005a; 84:1104–1116. [PubMed: 16304439]

Hajishengallis G, Tapping RI, Martin MH, Nawar H, Lyle EA, Russell MW, Connell TD. Toll-like receptor 2 mediates cellular activation by the B subunits of type II heat-labile enterotoxins. Infect Immun. 2005b; 73:1343–1349. [PubMed: 15731031]

Hajishengallis, G.; Russell, MW. Molecular approaches to vaccination against oral infections. In: Rogers, A., editor. Molecular Oral Microbiology. Norfolk, UK: Caister Academic Press; 2008. p. 257-285.

Holmes, R.; Jobling, MG.; Connell, T. Cholera toxin and related enterotoxins of gram-negative bacteria. Bacterial toxins and virulence factors in disease. In: Moss, J.; Iglewski, B.; Vaughn, M.; Tu, AT., editors. Handbook of natural toxins. Vol. 8. New York: Marcel Dekker, Inc; 1995. p. 225-255.

Holmgren J, Lycke N, Czerkinsky C. Cholera toxin and cholera B subunit as oral-mucosal adjuvant and antigen vector systems. Vaccine. 1993; 11:1179–1184. [PubMed: 8256498]

Holmgren J, Czerkinsky C, Eriksson K, Mharandi A. Mucosal immunisation and adjuvants: a brief overview of recent advances and challenges. Vaccine. 2003; 21(Suppl 2):S89–95. [PubMed: 12763689]

Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. Nat Med. 2005; 11(4 Suppl):S45–53. [PubMed: 15812489]

Holmgren, J.; Svennerholm, A-M. Mucosal immunity to bacteria. In: Mestecky, J.; Bienenstock, J.; Lamm, ME., et al., editors. Mucosal Immunology. 3. Amsterdam: Elsevier/Academic Press; 2005. p. 783-797.

Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. Nat Immunol. 2004; 5:987–995. [PubMed: 15454922]

Kolls JK, Linden A. Interleukin-17 family members and inflammation. Immunity. 2004; 21:467–476. [PubMed: 15485625]

Krueger J, Ray A, Tamm I, Sehgal PB. Expression and function of interleukin-6 in epithelial cells. J Cell Biochem. 1991; 45:327–334. [PubMed: 2045425]

Kwissa M, Kasturi SP, Pulendran B. The science of adjuvants. Expert Rev Vaccines. 2007; 6:673–684. [PubMed: 17931149]

la Sala A, He J, Laricchia-Robbio L, Gorini S, Iwasaki A, Braun M, Yap GS, Sher A, Ozato K, Kelsall B. Cholera toxin inhibits IL-12 production and CD8alpha+ dendritic cell differentiation by cAMP-mediated inhibition of IRF8 function. J Exp Med. 2009; 206:1227–1235. [PubMed: 19487420]

Lalli E, Sassone-Corsi P. Signal transduction and gene regulation: the nuclear response to cAMP. J Biol Chem. 1994; 269:17359–17362. [PubMed: 8021233]

Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, McClanahan T, Kastelein RA, Cua DJ. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. J Exp Med. 2005; 201:233–240. [PubMed: 15657292]

Lavelle EC, McNeela E, Armstrong ME, Leavy O, Higgins SC, Mills KH. Cholera toxin promotes the induction of regulatory T cells specific for bystander antigens by modulating dendritic cell activation. J Immunol. 2003; 171:2384–2392. [PubMed: 12928385]

Lavelle EC, Jarnicki A, McNeela E, Armstrong ME, Higgins SC, Leavy O, Mills KH. Effects of cholera toxin on innate and adaptive immunity and its application as an immunomodulatory agent. J Leukoc Biol. 2004; 75:756–763. [PubMed: 14704372]

Lee JB, Jang JE, Song MK, Chang J. Intranasal delivery of cholera toxin induces th17-dominated T-cell response to bystander antigens. PLoS One. 2009; 4:e5190. [PubMed: 19360100]

Liang S, Wang M, Tapping RI, Stepensky V, Nawar HF, Triantafilou M, Triantafilou K, Connell TD, Hajishengallis G. Ganglioside GD1a is an essential coreceptor for toll-like receptor 2 signaling in response to the B subunit of Type IIb enterotoxin. J Biol Chem. 2007a; 282:7532–7542. [PubMed: 17227759]

Liang S, Wang M, Triantafilou K, Triantafilou M, Nawar HF, Russell MW, Connell TD, Hajishengallis G. The A subunit of Type IIb enterotoxin (LT-IIb) suppresses the proinflammatory potential of the B subunit and its ability to recruit and interact with TLR2. J Immunol. 2007b; 178:4811–4819. [PubMed: 17404262]

Liang S, Hosur KB, Lu S, Nawar HF, Weber BR, Tapping RI, Connell TD, Hajishengallis G. Mapping of a microbial protein domain involved in binding and activation of the TLR2/TLR1 heterodimer. J Immunol. 2009a; 182:2978–2985. [PubMed: 19234193]

Liang S, Hosur KB, Nawar HF, Russell MW, Connell TD, Hajishengallis G. In vivo and in vitro adjuvant activities of the B subunit of Type IIb heat-labile enterotoxin (LT-IIb-B_5) from *Escherichia coli*. Vaccine. 2009b; 27:4302–4308. [PubMed: 19450646]

Liu H, Komai-Koma M, Xu D, Liew FY. Toll-like receptor 2 signaling modulates the functions of CD4+ CD25+ regulatory T cells. Proc Natl Acad Sci U S A. 2006; 103:7048–7053. [PubMed: 16632602]

Liu YJ, Banchereau J. Regulation of B-cell commitment to plasma cells or to memory B cells. Semin Immunol. 1997; 9:235–240. [PubMed: 9237929]

Luross JA, Heaton T, Hirst TR, Day MJ, Williams NA. *Escherichia coli* heat-labile enterotoxin B subunit prevents autoimmune arthritis through induction of regulatory CD4+ T cells. Arthritis & Rheumatism. 2002; 46:1671–1682. [PubMed: 12115200]

Lycke N, Tsuji T, Holmgren J. The adjuvant effect of Vibrio cholerae and Escherichia coli heat-labile enterotoxins is linked to their ADP-ribosyltransferase activity. Eur J Immunol. 1992; 22:2277–2281. [PubMed: 1381311]

Marinaro M, Staats HF, Hiroi T, Jackson RJ, Coste M, Boyaka PN, Okahashi N, Yamamoto M, Kiyono H, Bluethmann H, Fujihashi K, McGhee JR. Mucosal adjuvant effect of cholera toxin in mice results from induction of T helper 2 (Th2) cells and IL-4. J Immunol. 1995; 155:4621–4629. [PubMed: 7594461]

Martin M, Metzger DJ, Michalek SM, Connell TD, Russell MW. Comparative analysis of the mucosal adjuvanticity of the type II heat-labile enterotoxins LT-IIa and LT-IIb. Infect Immun. 2000; 68:281–287. [PubMed: 10603399]

Martin M, Metzger DJ, Michalek SM, Connell TD, Russell MW. Distinct cytokine regulation by cholera toxin and type II heat-labile toxins involves differential regulation of CD40 ligand on CD4(+) T cells. Infect Immun. 2001; 69:4486–4492. [PubMed: 11401990]

Martin M, Rehani K, Jope RS, Michalek SM. Toll-like receptor-mediated cytokine production is differentially regulated by glycogen synthase kinase 3. Nat Immunol. 2005; 6(8):777–784. [PubMed: 16007092]

Mendel I, Shevach Ethan M. The IL-10-producing competence of Th2 cells generated in vitro is IL-4 dependent. Eur J Immunol. 2002; 32:3216–3224. [PubMed: 12555667]

Mestecky J, Nguyen H, Czerkinsky C, Kiyono H. Oral immunization: an update. Curr Opin Gastroenterol. 2008; 24:713–719. [PubMed: 19122521]

Munoz E, Zubiaga AM, Merrow M, Sauter NP, Huber BT. Cholera toxin discriminates between T helper 1 and 2 cells in T cell receptor-mediated activation: role of cAMP in T cell proliferation. J Exp Med. 1990; 172:95–103. [PubMed: 2162906]

Murphy CA, Langrish CL, Chen Y, Blumenschein W, McClanahan T, Kastelein RA, Sedgwick JD, Cua DJ. Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. J Exp Med. 2003; 198:1951–1957. [PubMed: 14662908]

Nashar TO, Webb HM, Eaglestone S, Williams NA, Hirst TR. Potent immunogenicity of the B subunits of *Escherichia coli* heat-labile enterotoxin: receptor binding is essential and induces differential modulation of lymphocyte subsets. Proc Natl Acad Sci USA. 1996; 93:226–230. [PubMed: 8552610]

Nashar TO, Hirst TR, Williams NA. Modulation of B-cell activation by the B subunit of Escherichia coli enterotoxin: receptor interaction up-regulates MHC class II, B7, CD40, CD25 and ICAM-1. Immunology. 1997; 91:572–578. [PubMed: 9378497]

Nawar HF, Arce S, Russell MW, Connell TD. Mucosal adjuvant properties of mutant LT-IIa and LT-IIb enterotoxins that exhibit altered ganglioside-binding activities. Infect Immun. 2005; 73:1330–1342. [PubMed: 15731030]

Parry G, Mackman N. Role of cyclic AMP response element-binding protein in cyclic AMP inhibition of NF-κB-mediated transcription. J Immunol. 1997; 159:5450–5456. [PubMed: 9548485]

Petrovska L, Lopes L, Simmons CP, Pizza M, Dougan G, Chain BM. Modulation of dendritic cell endocytosis and antigen processing pathways by *Escherichia coli* heat-labile enterotoxin and mutant derivatives. Vaccine. 2003; 21:1445–1454. [PubMed: 12615441]

Phipps, Paula A.; Stanford, Miles R.; Sun, J-B.; Xiao, B-G.; Holmgren, J.; Shinnick, T.; Hasan, A.; Mizushima, Y.; Lehner, T. Prevention of mucosally induced uveitis with a HSP60-derived peptide linked to cholera toxin B subunit. European Journal of Immunology. 2003; 33:224–232. [PubMed: 12594851]

Pizza M, Domenighini M, Hol W, Giannelli V, Fontana MR, Giuliani MM, Magagnoli C, Peppoloni S, Manetti R, Rappuoli R. Probing the structure-activity relationship of Escherichia coli LT-A by site-directed mutagenesis. Mol Microbiol. 1994; 14:51–60. [PubMed: 7830560]

Pizza M, Giuliani MM, Fontana MR, Monaci E, Douce G, Dougan G, Mills KH, Rappuoli R, Del Giudice G. Mucosal vaccines: non toxic derivatives of LT and CT as mucosal adjuvants. Vaccine. 2001; 19:2534–2541. [PubMed: 11257389]

Raveney BJ, Richards C, Aknin ML, Copland DA, Burton BR, Kerr E, Nicholson LB, Williams NA, Dick AD. The B subunit of *Escherichia coli* heat-labile enterotoxin inhibits Th1 but not Th17 cell responses in established experimental autoimmune uveoretinitis. Invest Ophthalmol Vis Sci. 2008; 49:4008–4017. [PubMed: 18469197]

Ryan EJ, McNeela E, Pizza M, Rappuoli R, O'Neill L, Mills KH. Modulation of innate and acquired immune responses by *Escherichia coli* heat-labile toxin: distinct pro- and anti-inflammatory effects of the nontoxic AB complex and the enzyme activity. J Immunol. 2000; 165:5750–5759. [PubMed: 11067933]

Sad S, Marcotte R, Mosmann TR. Cytokine-induced differentiation of precursor mouse CD8+ T cells into cytotoxic CD8+ T cells secreting Th1 or Th2 cytokines. Immunity. 1995; 2:271–279. [PubMed: 7697544]

Satchell KJ. Activation and suppression of the proinflammatory immune response by *Vibrio cholerae* toxins. Microbes Infect. 2003; 5:1241–1247. [PubMed: 14623020]

Simons K, Toomre D. Lipid rafts and signal transduction. Nat Rev Mol Cell Biol. 2000; 1:31–39. [PubMed: 11413487]

Sun JB, Li BL, Czerkinsky C, Holmgren J. Enhanced Immunological Tolerance against Allograft Rejection by Oral Administration of Allogeneic Antigen Linked to Cholera Toxin B Subunit. Clinical Immunology. 2000; 97:130–139. [PubMed: 11027453]

Sun JB, Rask C, Olsson T, Holmgren J, Czerkinsky C. Treatment of experimental autoimmune encephalomyelitis by feeding myelin basic protein conjugated to cholera toxin B subunit. Proc Natl Acad Sci (USA). 1996; 93:7196–7201. [PubMed: 8692968]

Takahashi I, Marinaro M, Kiyono H, Jackson RJ, Nakagawa I, Fujihashi K, Hamada S, Clements JD, Bost KL, McGhee JR. Mechanisms for mucosal immunogenicity and adjuvancy of *Escherichia coli* labile enterotoxin. J Infect Dis. 1996; 173:627–635. [PubMed: 8627026]

Triadafilopoulos G, Pothoulakis C, Weiss R, Giampaolo C, Lamont JT. Comparative study of *Clostridium difficile* toxin A and cholera toxin in rabbit ileum. Gastroenterology. 1989; 97:1186–1192. [PubMed: 2551764]

Triantafilou M, Miyake K, Golenbock DT, Triantafilou K. Mediators of innate immune recognition of bacteria concentrate in lipid rafts and facilitate lipopolysaccharide-induced cell activation. J Cell Sci. 2002; 115:2603–2611. [PubMed: 12045230]

Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat Rev Immunol. 2003; 3:133–146. [PubMed: 12563297]

van den Akker F, Sarfaty S, Twiddy EM, Connell TD, Holmes RK, Hol WG. Crystal structure of a new heat-labile enterotoxin, LT-IIb. Structure. 1996; 4:665–678. [PubMed: 8805549]

van Loon FP, Clemens JD, Chakraborty J, Rao MR, Kay BA, Sack DA, Yunus M, Ali M, Svennerholm AM, Holmgren J. Field trial of inactivated oral cholera vaccines in Bangladesh: results from 5 years of follow-up. Vaccine. 1996; 14:162–166. [PubMed: 8852414]

Weaver CT, Hatton RD, Mangan PR, Harrington LE. IL-17 family cytokines and the expanding diversity of effector T cell lineages. Annu Rev Immunol. 2007; 25:821–852. [PubMed: 17201677]

Wilson AD, Robinson A, Irons L, Stokes CR. Adjuvant action of cholera toxin and pertussis toxin in the induction of IgA antibody response to orally administered antigen. Vaccine. 1993; 11:113–118. [PubMed: 8382416]

Yamamoto M, Kiyono H, Yamamoto S, Batanero E, Kweon MN, Otake S, Azuma M, Takeda Y, McGhee JR. Direct effects on antigen-presenting cells and T lymphocytes explain the adjuvanticity of a nontoxic cholera toxin mutant. J Immunol. 1999; 162:7015–7021. [PubMed: 10358143]

Yura M, Takahashi I, Terawaki S, Hiroi T, Kweon M-N, Yuki Y, Kiyono H. Nasal administration of cholera toxin (CT) suppresses clinical signs of experimental autoimmune encephalomyelitis (EAE). Vaccine. 2001; 20:134–139. [PubMed: 11567757]

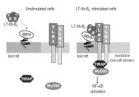

Figure 1. Model for ganglioside-TLR cooperation for cell activation by LT-IIb-B_5

Upon binding of LT-IIb-B_5 to GD1a, GD1a facilitates the interaction of LT-IIb-B_5 with the TLR2/TLR1 signaling complex, which is recruited to lipid rafts. Induction of TLR2/TLR1 signaling for NF-κB activation by LT-IIb-B_5 occurs at the cell surface and requires the adaptor proteins TIRAP and MyD88, which colocalize with the LT-IIb-B_5 receptor complex (GD1a/TLR2/TLR1) (Hajishengallis et al. 2005b; Liang et al. 2007a; Liang et al. 2009b).

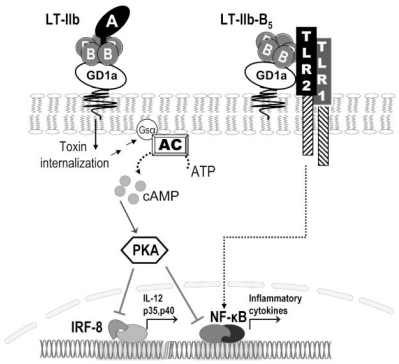

Figure 2. Differential and antagonistic effects on proinflammatory cytokine induction by LT-IIb holotoxin and its B pentamer

LT-IIb-B_5 activates the TLR2/TLR1 heterodimer and induces NF-κB-dependent production of proinflammatorry cytokines (Liang et al. 2009a; Liang et al. 2007a; Liang et al. 2007b). In contrast, the holotoxin does not interact with TLR2/TLR1 due to A subunit-dependent steric hindrance (Liang et al. 2007b; Liang et al. 2009). However, upon GD1a binding and internalization of the holotoxin, the ADP-ribosyltransferase activity of its A subunit activates the Gsα component of adenylate cyclase (AC). This leads to elevation of intracellular cAMP, activation of cAMP-dependent PKA, and inhibition of NF-κB-dependent transcription of proinflammatory cytokines (*e.g.*, TNF-α) (Liang et al. 2007b). This cAMP-dependent antagonistic action is not restricted to LT-IIb, since both CT and LT-IIa can similarly inhibit LT-IIb-B_5-induced cell activation (Hajishengallis et al. 2004). The cAMP signaling pathway was shown to also inhibit IL-12 expression through suppression of the interferon regulatory factor-8 (a critical transcription factor for IL-12 p35 and p40 gene expression) (la Sala et al. 2009). It is possible that, in addition to CT (la Sala et al. 2009), other cAMP-inducing enterotoxins can also inhibit IL-12 transcription.

Table 1

Functional comparison of heat-labile AB_5-type enterotoxins

		CT	LT-I	LT-IIa	LT-IIb
Receptors	Gangliosides	GM1	GM1	GD1b > GD1a > GM1	GD1a
	TLRs	−	−	TLR2/TLR1 (B subunit only)	TLR2/TLR1 (B subunit only)
Proinflammatory cytokine induction in macrophages		−	−	+ (B subunit only)	+ (B subunit only)
cAMP-dependent inhibition of inflammatory cytokines		+	+	+	+
In vivo anti-inflammatory action		+	ND	ND	ND
Inhibition of autoimmunity		+ (B subunit also)	+ (B subunit also)	ND	ND
In vivo adjuvanticity		+	+	+ (B subunit also)	+ (B subunit also)
A subunit-dependent but cAMP-independent adjuvant action		+*	+*	+**	+**
T-helper bias		Th2, Treg or Th17***	Th1, Th2	Th1, Th2	Th1 > Th2
Upregulation of APC costimulatory molecules and MHC class II		+	+	− (+ for B subunit)	− (+ for B subunit)
$CD8^+$ T-cell apoptosis		+	+	+	−
Suppression of CD40-CD40L interactions		+	+	−	−

* Catalytically defective mutants (point mutations in the A subunit).

** Mutants with abrogated cAMP-inducing activity due to altered ganglioside binding (point mutations in the B subunit).

*** Promotion of Th17, rather than Treg, depends on IL-6 induction in addition to TGF-β.

ND, not determined.