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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

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Abstract

The heat-labile enterotoxins (HLT) expressed by *Vibrio cholerae* (cholera toxin) and *Escherichia coli* (LT-I, LT-IIa, and LT-IIb) are potent systemic and mucosal adjuvants. Co-administration of the enterotoxins with a foreign antigen (Ag) produces an augmented immune response to that antigen. Although each enterotoxin has potent adjuvant properties, the means by which the enterotoxins induce various immune responses are distinctive for each adjuvant. Various mutants have been engineered to dissect the functions of the enterotoxins required for their adjuvanticity. The capacity to strongly bind to one or more specific ganglioside receptors appears to drive the distinctive immunomodulatory properties associated with each enterotoxin. Mutant enterotoxins with ablated or altered ganglioside binding affinities have been employed to investigate the role of gangliosides in enterotoxin-dependent immunomodulation.

Keywords

enterotoxin; adjuvant; immunomodulation; ganglioside; signal transduction

INTRODUCTION

Heat-labile enterotoxins (HLT) are potent mucosal and systemic adjuvants. Yet, only in the last ten years have detailed investigations begun to unravel the events by which HLTs augment cellular and humoral immune responses to foreign antigens (Ag). These experiments have revealed that HLTs stimulate the immune system using a series of complex mechanisms [1]. A plethora of diverse interactions between HLT and various immunocompetent cell types [2] and a multitude of cellular responses elicited in those cell types have been identified. Some of these immunomodulatory activities are believed to be induced, either directly or indirectly, as a response to dramatic increases in the intracellular cAMP levels conferred by the toxic (catalytic) activities of the HLT [3-10]. cAMP is a strong secondary messenger molecule employed by many types of cells to regulate gene expression. On the other hand, not all immune responses elicited by HLTs ensue from the enterotoxins' catalytic properties [7,11,12]. Agents that raise cAMP levels in the cell (e.g. forskalin) do not elevate immune responses to Ags [13]. Binding of HLTs to their specific receptors on the surfaces of immunocompetent cells is often sufficient to trigger signal transduction events [14,15] which are likely required for immune responsiveness and immunomodulation. Recent studies have shown that binding of

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cholera toxin (CT), an HLT produced by *Vibrio cholerae*, to specific cell surface receptors on immunocompetent cells promotes redistribution of components of the plasma membrane within lipid microdomains (rafts) involved in signal transduction [16]. Signals which are promulgated by binding of HLTs to their receptors foster up-regulation and down-regulation expression of a number of genes that encode essential immune functions [1]. The processes by which immunomodulatory activities are promoted by binding of HLTs to receptors have not been fully characterized or enumerated. Recent investigations using mutant HLTs with altered receptor binding activities, however, have revealed a number of mechanisms by which HLTs likely augment immune responses. This review will concentrate on those mutant HLTs, the immune responses which are (or are not) induced by those mutants, and the receptors in the plasma membrane which are involved in productive HLT-immunocompetent cell interactions.

Gangliosides

Gangliosides are members of an extensive family of sialylated glycosphigolipids which are ubiquitously expressed on the plasma membrane of most, if not all eukaryotic cells [17]. Structurally, gangliosides are oligoglycosylceramides which contain *N*-acetylneuraminic acid (sialic acid or NeuAc) residues or less commonly *N*-glycolyl-neuraminic acid (NeuGc) joined via glycosidic linkages to one or more of the monosaccharide units [18]. The ceramide core is usually embedded in the lipid bilayer of the cell while the sugar groups are normally exposed to the surface of the cell. The diversity in the family arises from the various arrangements by which the sugar groups are arranged on the ceramide core [18](Fig. 1). This diversity is reflected in the distribution of gangliosides in nature. The types of gangliosides vary immensely both between different species and between different cell types within a single species (e.g., resting T cells are enriched in ganglioside GM3 (GM3) and ganglioside GD1a (GD1a)[19, 20]; B cell neoplasms were shown to predominate in ganglioside GM2 (GM2)[21]; mouse macrophages express GD1a, ganglioside GM1b, and ganglioside GM1a, in combination with a population of minor gangliosides [22]). Interestingly, gangliosides are not always found inserted into plasma membranes. Human serum contains levels of GD1a near to that ganglioside's critical micellar concentration [23,24]. While gangliosides are best known as receptors for numerous bacterial toxins [25], these oligoglycosylceramides also have strong bioactive roles in normal cellular metabolism. Many gangliosides participate, directly or indirectly, in regulating responses of cells. For example, gangliosides have been established as receptors for platelet-derived growth factor, epidermal growth factor, and insulin [26,27]. Other gangliosides have immunosuppressive effects on T cells, B cells, and macrophages [23,28-31]. Immunomodulatory responses in those immunocompetent cells are likely induced by signals which are translated across the membrane after binding of ligands to gangliosides [26,32-35].

Crosslinking of gangliosides

Whereas binding of a ligand to a single ganglioside in the plasma membrane may be sufficient to trigger certain cellular responses, it is likely that ligands which have the capacity to crosslink gangliosides are powerful agents for inducing responses in some immunocompetent cells [36]. Crosslinking of ganglioside GD3 (GD3) with an anti-GD3 monoclonal antibody induced proliferation of T cells, secretion of interferon-γ (IFN- γ) and interleukin-6 (IL-6), and increased expression of HLA class II molecules and interleukin-2 (IL-2) receptors [37-39]. Hammond et al. [16] hypothesized that clustering of a small number of lipids in a cell's membrane potentially could reorganize many other membrane components in a rapid manner. When artificial lipid vesicles were treated with the B pentamer of CT (CTB) which has the capacity to bind to five GM1 gangliosides (see below), the GM1 gangliosides in the vesicles were crosslinked. Crosslinking of GM1 induced phase-separation of the lipids within the vesicles which was correlated with a dramatic redistribution of a transmembrane peptide [16]. Crosslinking of ganglioside GM1 (GM1) in Jurkat T cells was correlated with

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phosphorylation of phospholipase C, a signaling molecule found within lipid-rich domains (rafts) of the plasma membrane [40]. CTB also was capable of substituting for co-stimulation upon activation of T cells, presumably by signaling after crosslinking GM1 in rafts [41] . It would be predicted, therefore, that any molecule which interacts with a ganglioside on the surface of an immunocompetent cell has the potential to elicit one or more immunomodulatory responses. The HLT produced by *Escherichia coli* and *Vibrio cholera*e which have five ganglioside-binding site, can crosslink gangliosides [42]. Both CT and LT-I strongly exhibit immunostimulatory properties [1,43-45].

Type I and Type II heat-labile enterotoxins

The HLT of *E. coli* and *Vibrio cholerae* belong to a family of structurally-related proteins that induce diarrheal symptoms in humans and animals [46]. The family is divided into two major groups based on genetic, biochemical, and immunological characteristics [46](Fig. 2). The Type I subfamily consists of CT from *V. cholerae*, the LT enterotoxin (referred herein as LT-I) of *E. coli*, and antigenically-related enterotoxin from several other enteric bacteria [46]. The Type II HLT subfamily is comprised of LT-IIa enterotoxin of *E. coli*, and LT-IIb, its partially cross-reacting antigenic variant [46]. The toxicity of Type I and Type II enterotoxins is conferred by an endogenous enzymatic activity which catalyzes an ADP ribosylation of the G_{sa} subunit of the adenylate cyclase complex in the plasma membrane of enterocytes and other cells [47-49]. Ribosylation of the G_{sa} subunit stimulates runaway synthesis of cAMP in the cell which in turn initiates a series of cellular events, one of which is a major change in transmembranal ion flux [46,48]. Type I and Type II heat-labile enterotoxins are oligomeric proteins composed of a single A polypeptide which is non-covalently bound to a pentameric array of B polypeptides [46,50]. Fragment A1, derived by proteolytic cleavage and reduction of an intrachain disulfide bond in the A polypeptide, is the toxic moiety, while the A2 fragment interacts in a non-covalent manner with the B pentamer to promote holotoxin assembly [47, 50]. Binding of the enterotoxins to specific receptors on the plasma membrane of target cells is mediated solely by the B polypeptides [47,51]. Structural constraints to maintain toxic activity has highly conserved the amino acid sequences of the A polypeptides of the Type I and Type II HLT [46]. The B polypeptides are less conserved. While the B polypeptides of CT and LT-I exhibit over 80% identity at the amino acid level, the B polypeptides of LT-IIa and LT-IIb have little homology (<14%) to the B polypeptides of CT or LT-I, or to each other [46].

The receptors for CT, LT-I, LT-IIa, and LT-IIb are one or more gangliosides which are bound by the B pentamers of the HLT [42,51]. Each B pentamer has five binding sites for its respective ganglioside receptor, thus enabling the holotoxin to crosslink multiple gangliosides. The divergence in amino acid sequences of the B polypeptides of CT, LT-I, LT-IIa, and LT-IIb confers distinctive ganglioside binding patterns on the HLT [46]. CT and LT-I bind strongly to to ganglioside GM1. LT-I, however, also has affinity for polyglycosylceramides, asialo-GM1, GM2, and polylactosamine-containing glycoproteins [52-54]. LT-IIa and LT-IIb exhibit a more divergent pattern of ganglioside specificity. LT-IIa binds most avidly to ganglioside GD1b (GD1b), has lesser binding avidity for GD1a, and has a low but measurable avidity for GM1 [51]. LT-IIb binds with high affinity only to GD1a [51], but also binds weakly to several other minor and unidentified gangliosides (T.D.C. and Hesham F. Nawar, unpubl.). It is thought that the enterotoxins evolved different ganglioside-binding activities and specificities as a means to bestow host specificity in respect to their ability to intoxicate particular animal species, tissues or cell types [55-58]. It is becoming clear, however, that these differences in ganglioside-binding activities also dictate the types of immunocompetent cells to which these HLT can bind, and at least in part, determine the cellular and molecular pathways within those immunocompetent cells which the four HLT stimulate to augment immune responses to co-administered Ags.

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Adjuvant properties of HLT

The capacities of CT and LT-I to augment immune responses to co-administered Ags have been reviewed elsewhere [1,8,44,45]. Thus, only the most important features relating to the immunomodulating activities of those two HLT will be summarized here. Northrup and Fauci [59] initially reported that CT, when delivered by the intravenous route with a foreign Ag, behaved as an adjuvant. Subsequently, Elson and Ealding [60] detected adjuvant activity when CT was administered by an alternate route of administration (*per oris*). It was noted in both studies that the adjuvant effects were observed only when CT and the Ag were administered simultaneously. The ability of CT to augment immune responses was not limited to short term effects. Reactivity to Ag, and to CT, was detectable long after the initial immune response. These data indicated that CT also augmented long-term memory responses to a co-administered Ag [61,62]. While the precise mechanism(s) by which CT exerts its adjuvant effect is still unclear, the response is usually predominantly correlated with a Th2-type polarization, a model that is supported by measurements of cytokine production and antibody isotype expression [63-66]. After immunization with CT as an adjuvant, interleukin 4 (IL-4) levels, a cytokine needed for B cell differentiation, is significantly elevated in gut-associated tissues and in spleen, while the levels of interferon gamma (INF-g), a cytokine needed to evoke cellular immune responses, either decreased or remained static [67,68]. CT also selectively inhibited production of IL-2 and IFN-γ from Th1 cells while increasing the production of IL-4 and IL-5 from Th2 cells [67,68]. *per oris* administration of CT predominately induced production of immunoglobulin G1 (IgG1) and secretory IgA (SIgA) in immunized animals [66,67]. CT was also reported to enhance production of IgG, IgE, and IgA in animal models [69,70]. When cultured Ag-specific lymph node cells were treated with LT-I, an increased activation of B cells and CD4+ T lymphocytes reflected in increased CD25 expression, was observed with a concomitant complete depletion of CD8+ lymphocytes [71]. Supernatants from the mixed lymphocytes cultures treated with LT-I contained no IL-4, IL-5, and IL-12, had decreased IFN-γ, but contained increased amounts of IL-2. This distribution of cytokines likely reflected the activation status of B cells and CD4+ T cells in the LT-I-treated population [71]. Recent investigations by Arce et al. [72] demonstrated that the Th2-like immunomodulating activities of CT are not due solely to its effects on T cells. In splenic populations, CT facilitates differentiation of naive B cells into plasma cells [72]. This B cell response was also T cell-dependent. Facilitated differentiation of B cells required the presence of CT-treated T cells in the culture [72]. While many of the effects of CT and LT-I are observed in B cells and T cells, the immunomodulating properties of CT and LT-I have also be attributed to direct effects on lymphoreticular cells. After CT administration, Ag-presentation was enhanced in macrophages [73]. MHC class II molecule expression and peptide presentation by macrophages was reported to be influenced by CT [73]. Several studies demonstrated that treatment with CT induced maturation and mobilization of DCs [74-76]. Dendritic cells (DC) treated with CT produced and secreted IL-1 [77]. CT-treated DC also up-regulated expression of HLA-DR, B7.1, and B7.2, and primed naive CD4+CD45RA+ T cells in vitro, thus driving those cells toward a Th2 phenotype [63]. CT also inhibited synthesis of IL-12p70 and TNF-α, RANTES, MIP-1α, and MIP-1β by lipopolysaccharide-activated or CD40 ligand-activated DC [63]. Human monocyte-derived DC are matured by both CT and LT-I which up regulate expression of CD80, CD83, CD86, and HLA-DR in a cAMP-dependent manner [78]. Animal studies showed that treatment with CT had a positive effect on uptake of models Ags from the lumen of the gut, presumably by affecting the efficiency of Ag-presenting cells [79]. Yet, while CT and LT-I generally induce Th2-type immune responses, there is gathering data that both Type I enterotoxins stimulate Th1 responses. Cytotoxic lymphocytes were evoked in several different immunization models when either CT or LT-I were employed as mucosal adjuvants to a bystander antigen ([80-83]. In fact, even the B pentamer of CT was shown to induce genital antigen-specific CTLs in a murine model after intravaginal so-immunization with the antigen [84]. Furthermore, treatment of dendritic cells with CT lead to the induction of cytotoxic CD8+ T cells in a B7.1-dependent

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manner [85]. Administration of CT-treated DC was correlated with rejection of an antigen-specific tumor in mice which required CD8+ T cells [86]. CT also appears to have a capacity for inducing regulatory T cells by modulating DC activation [87]. The mechanisms by which the other HLTs promote development of regulatory T cells have yet to be fully described [87].

Detoxified HLT

While it is evident that CT and LT-I are potent adjuvants, their inherent toxicities have precluded their use as adjuvants in human vaccines [1]. This omission is particularly relevant for mucosal vaccines. Mouse models demonstrated that CT and LT-I, when administered via the intranasal route, efficiently bound to the nasal neuroepithelium and subsequently trafficked by retrograde transport along the underlying olfactory nerves to the olfactory bulbs in the brain [88,89]. Retrograde transport of CT likely exerted an inflammatory effect on brain tissues. After intranasal administration of CT, transcriptional expression of the proinflammatory mediators IL-1β, TNF-α, COX-2, MCP-1, MIP-1α, and cyclooxygenase-2 were all elevated in the murine brain [90]. Retrograde transport of CT and LT-I require binding to GM1 since mutant enterotoxins (below) having no detectible binding to GM1 did not elicit the inflammatory effects [90]. As expected, these observations raised serious concerns for the use of CT and LT-I in vaccines, a consideration that appeared to be justified after a significant number of cases of Bell's palsy, a form of temporary facial paralysis resulting from damage or trauma to one of the two facial nerves, was detected in individuals who had been administered an intranasal LT-I-containing vaccine against influenza virus [91]. In an attempt to produce an HLT in which the potentially serious toxicities were ablated, recombinant engineering was employed to produce non-toxic forms of CT and LT-I. In initial attempts, mutant holotoxins were engineered which targeted single-point amino acid substitutions at amino acids in the A polypeptides which were considered to be critical for ribosylation activity. While many of these mutant holotoxins [e.g., CT(E29H)[92] LT-I(H44A)[93], LT-I(A69G)[3], LT-I(S61F)[94, 95], LT-I(S63K)[10], LT-I(S63Y)[96], and LT-I(A72R)[97], LT-I(E112K)[94,95]] (mutants will be noted by the single letter code for the amino acid in wt HLT, the position of the amino acid in the polypeptide, and the single letter code for the substituted amino acid) retained adjuvant activities, further evaluation demonstrated that mutant enterotoxins CT(E29H), LT-I(H44A), LT-I(A72R), and LT-I(A69G) retained residual enzymatic activity. Additional mutant enterotoxins were engineered to alter other properties of the enterotoxins required for full toxic activity including LT-I(R192G)[9], a mutant holotoxin in which a proteolytically-sensitive site within the A2 domain of the A polypeptide required for full toxicity was disrupted with a glycine for arginine substitution at amino acid position 192 of the A polypeptide. This mutant enterotoxin, which exhibited reduced capacity to increase intracellular cAMP [3], had adjuvant properties similar to that of wt LT-I [9].

Non-toxic B pentamers

Although mutant holotoxins LT-I(S61F), LT-I(E112K), LT-I(S63Y) and others exhibited diminished capacity to increase levels of cAMP within cells, there was continuing concern that these mutant HLT retained residual capacity to affect cellular cAMP production that was below the level of detection of the current assays or that the non-immunocompetent cell types which were usually employed in the bioassays to measure cAMP accumulation were irrelevant, i.e. cAMP accumulation and the respective cAMP-dependent responses induced by the mutant holotoxins might differ in immunocompetent cells. These concerns led to experiments to determine whether the non-toxic B pentamers of HLT exhibited adjuvant properties. In the absence of the A polypeptides, B pentamers of the HLT (CTB and LT-IB) exhibit no detectable toxicity in standard Y-1 adrenal cell bioassays [3]. While usually not as potent as the respective holotoxins, multiple studies established that CTB and LT-IB has potent adjuvant immunomodulatory activity in vivo. For example, a strong serum IgG antibody response

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against influenza HA was obtained in rabbits after immunization with the Ag in the presence of CTB [98,99]; immunization of mice with the SBR Ag of *Streptococcus mutans* coupled to CTB stimulated a potent anti-SBR SIgA response [100]; immunization of mice with herpes simplex virus glycoproteins in the presence of LT-IB potentiated antibody and T cell responses that correlated with protection against ocular HSV-1 infection [101]; and, CTB used as an intranasal adjuvant induced protective responses against challenge with influenza virus [102-104]. Treatment with CTB also enhanced the Ag-presenting function of macrophages [73]. Although proinflammatory cytokines in the olfactory bulbs were not measured, neither CTB nor LT-IB used at 0.1μg/dose/mouse caused histological changes in the brain of intranasally immunized mice [105]. And, CTB and LT-IB have been shown to promoter tolerance to antigens when administered orally (for a review, see [106]). These studies, and many others, provided strong evidence that CTB and LT-IB alone, at least in combination with particular Ags and administered by particular routes, often exhibited adjuvant properties [1, 9,44,45,107]. These studies also provided strong evidence that binding to GM1 was critically important in the immunostimulatory properties of CT and LT-I. Yet, the importance of GM1 binding for the adjuvant properties of CT and LT-I have only been intensively evaluated in the last few years.

Mutant Type I HLTs with altered ganglioside binding activities

GM1 is ubiquitously distributed in the cells of most mammals (i.e. no animals or cell lines were available that lacked expression of GM1) [108-110]. Thus, it has been difficult to design experiments to directly evaluate the correlation between CT or LT-I and GM1 binding, adjuvanticity, and immunomodulation. In 1991, however, mutant CT holotoxins having single-point substitutions at amino acid position 33 in the B polypeptide were engineered [111,112]. Of these mutant holotoxins, CT(G33D) has been the most extensively studied. Structural studies demonstrated that the G33D amino acid substitution had no affect either on pentameric assembly of the mutant B polypeptides or on non-covalent association of the mutant pentamers with A polypeptide to form holotoxin [111,112]. The mutant holotoxin also exhibited a decreased capacity to intoxicate cells, an effect presumably due to the inability of the mutant holotoxin to bind strongly to GM1 on the cell surface [111,112]. Similar G33D mutants of LT-I exhibited binding and toxic characteristics analogous to those of CT(G33D)[71,113]. Recognizing their potential as agents for dissecting the association between immunomodulation and GM1 binding, CTB(G33D) and LT-IB(G33D) holotoxins and B pentamers were employed by a bevy of investigators to determine if the immunomodulatory activities of CT and LT-I were dependent upon binding to GM1. In the majority of cases, abrogation of GM1-binding activity completely abolished, in vivo and in vitro, the immunomodulatory properties of CT and LT-I. For example, lymphocyte cultures treated with the wt B pentamer of LT-I (LT-IB) displayed an increase in the proportion of B cells [71], many of which expressed CD25, CD40, B7, ICAM-1, and MHC II, all of which indicated an activated state required for increased immune responsiveness [114]. Treatment with LT-B induced a complete depletion of CD8+ T cells by apoptosis, an increase in activation of CD4 + T cells, elevated production of IL-2, IL10, and IL-6, and decreased production of IL-12 and IFN-γ, all of which would tend to polarize the cells to a Th2-type of immune response [115, 116]. LT-I triggered activation of caspase 8 and caspase 3 in CD8+ T cells by a Fas and Tumor Necrosis Factor (TNF) receptor-independent process [117]. None of these effects, however, was observed when lymphocyte cultures were treated with the B pentamer of LT-I(G33D) [71,113,114,116-121]. The potent adjuvant properties of LT-IB were also altered by the G33D mutation. In comparison to the antibody titers of mice immunized with wt LT-IB, mice subcutaneously immunized with LT-IB(G33D) exhibited a 160-fold decrease in anti-LT-IB antibody titer [71]. Treatment of human CD14+ monocytes with LT-IB, but not with LT-IB (G33D) triggered release of IL-10 and IL-6, two B cell modifiers [115,122,123], and inhibited expression of IL-12 [CTB, however, exerted no effects on IL-12 production, a cytokine which

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polarizes T cells to a Th1-type of immune response [115], suggesting that these two closely related enterotoxins have unique immunomodulatory properties]. To determine if more potent adjuvant properties would be demonstrated by LT-I holotoxin containing the G33D mutation in the B polypeptides, mice were immunized with LT-I(G33D) holotoxin. Surprisingly, the LT-I(G33D) holotoxin exhibited residual toxic activity in a mouse Y-1 adrenal cell bioassay and stimulated amounts of cAMP in Caco-2 cells equivalent to those stimulated by treatment with wt LT-I [113]. These data suggested that the amino acid substitution at G33 in the B polypeptide of LT-I did not fully abrogate binding of the mutant enterotoxin to GM1. LT-I (G33D) holotoxin, when employed as an oral adjuvant, was also incapable of augmenting the levels of IgG or IgA directed against a co-administered Ag or against the holotoxin [113]. Collectively, these data indicated that strong binding of LT-I or CT to GM1 was essential for augmenting the enterotoxins' immune responses.

Adjuvant properties of Type II HLT

Experiments using the G33D mutants confirmed that strong binding to GM1 was a key event in the immunomodulatory capacities of LT-I and CT by inducing or suppressing relevant immune responses in various immunocompetent cell types. These observations, however, stimulated a related question. Are the immunostimulatory effects solely dependent upon binding to GM1? This question has been addressed by evaluating the adjuvant properties of LT-IIa and LT-IIb and the immunomodulatory properties of their respective ganglioside binding mutants [2,124-130]. Although the immunomodulatory properties of LT-IIa and LT-IIb have not been as extensively investigated as those of CT and LT-I, recent studies have demonstrated that the mucosal and systemic adjuvant properties of LT-IIa and LT-IIb are equivalent to those of CT and LT-I [2,124-130]. Yet, LT-IIa and LT-IIb exhibited unique immunological properties [2,124-130]. While the capacities of LT-IIa and LT-IIb to augment immune responses to foreign Ags were similar, the immune responses and immunomodulatory events elicited by LT-IIa and LT-IIb in mouse immunization models and in cell cultures were very distinguishable from those evoked by CT or LT-I, indicating that the Type II HLT were unique immunostimulants [2,124-130]. Cytokine and antibody isotype distributions indicated that LT-IIa and LT-IIb displayed a trend toward eliciting a more balanced Th1/Th2 response, in contrast to the predominant Th2-like responses evoked by CT and, in most cases, by LT-I [2,126-130]. Evaluations of anti-CD3-stimulated human peripheral blood mononuclear cells (PBMC) demonstrated that CT suppressed production of IL-2, TNF-α, and IL-12 to a much greater degree than did either LT-IIa or LT-IIb [2,128]. In contrast to CT, LT-IIa and LT-IIb exerted no effects on expression of CD25 and CD69 by CD4+ T cells [72]. And CT, but not LT-IIa or LT-IIb, reduced expression of CD40 ligand on CD4+ T cells [128]. Treatment of CD4+ T cells with LT-IIa or LT-IIb elicited greater amounts of TNF-α and IL12p70 by monocyte-derived DCs than did treatment with CT [128]. Apoptotic effects were also disparate between the Type I and Type II enterotoxins. LT-IIa and CT, but not LT-IIb, induced apoptosis of CD8+ T cells [2]. Unlike CT, however, neither LT-IIa nor LT-IIb inhibited mitogen-driven CD4+ T cell proliferation [2]. LT-IIa or LT-IIb, in contrast to CT, did not facilitate rapid differentiation of B cells into plasma cells [72]. Finally, a chimeric LT-IIa holotoxin composed of the SBR Ag of *S. mutans* genetically fused to the A2 assembly domain of the A polypeptide of LT-IIa and assembled with the wt LT-IIa B pentamers, while eliciting strong anti-SBR antibody titers when used as an immunogen, did not up-regulate expression of B7.1 or B7.2 in B220+, CD11b+, or CD11c+ splenic cells [126]. In contrast, an SBR-A2/CTB chimera significantly elevated expression of those co-stimulatory receptors on those cell types [126]. Clearly, LT-IIa and LT-IIb must elicit their adjuvant effects by mechanisms distinctive from those utilized by either CT or LT-I, and the mechanisms by which LT-IIa and LT-IIb stimulate the immune system must be relatively novel since neither LT-IIa nor LT-IIb evoke the cytokine, apoptotic, proliferative, or cell differentiation pathways which are evoked by CT and LT-I [2,72,126-130].

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Mutant Type II HLT with altered ganglioside-binding properties

The current hypothesis is that the divergent patterns of immune responses evoked by LT-IIa and LT-IIb are due to their capacity to bind to non-GM1 ganglioside receptors which presumably induce different signal transduction pathways, elicit divergent changes in the plasma membrane, and/or differentially induce or suppress various intracellular responses of one or more types of immunocompetent cells. To test that hypothesis, mutant LT-IIa and LT-IIb holotoxins were engineered with single-point substitutions in their B polypeptides which altered their ganglioside-binding patterns [55-57]. Co-administration of mice by the intranasal route with AgI/II from *S. mutans* and LT-IIa(T34I), a mutant HLT which had no detectible binding activity by ganglioside-dependent ELISA for GD1b, GD1b, or GM1, the gangliosides most strongly bound by LT-IIa, failed to induce production of anti-Ag/II antibodies in either serum or saliva [130]. The failure of LT-IIa(T34I) to induce an immune response was likely due to the failure of the mutant enterotoxin to interact with one or more types of immunocompetent cells; LT-IIa(T34I) had no affinity for any class of cervical lymph node lymphocytes [130]. This failure to bind to the cells is reflected in the decreased affinity of LT-IIa(T34I) to gangliosides in vitro [130] which likely affected the mutant enterotoxin's toxic capacity. LT-IIa(T34I) exhibited little toxicity in a Y1 adrenal cell assay, nor did it increase cAMP levels in murine macrophages above the levels produced by treatment of the cells with the non-toxic LT-IIa B pentamer [129,130]. LT-IIa(T34I), however, did have some adjuvant properties. Boosting mice which had initially received AgI/II and LT-IIa(T34I) as a mucosal adjuvant with AgI/II alone had primed the mice for a strong Ag-specific humoral memory response [129]. A second set of LT-IIa mutants having single-point amino acid substitutions at amino acid position 14 in the B polypeptides revealed that toxicity was not only separable from adjuvanticity, but that adjuvanticity was directly correlated with strength of binding to gangliosides [130]. By ganglioside-dependent ELISA using purified bovine brain gangliosides, LT-IIa(T14S) and wt LT-II had equivalent affinities for GD1b, GD1a, and GM1 (and to the minor receptors GM2, GM3, anGQ1b, and GT1b) [130]. LT-IIa(T14I) and LT-IIa(T14D), however, bound to those same gangliosides with less avidity [LT-IIa > LT-II(T14S) > LT-II (T14I) >> LT-IIa(T14D)] [130]. When administered via the intranasal route, mutants LT-IIa (T14S), LT-IIa(T14I), and LT-IIa(T14D) all induced strong mucosal and systemic immune responses to the co-administered Ag which were equivalent to the responses induced by use of wt LT-IIa [130]. While LT-IIa(T14S) was capable of eliciting high levels of intracellular cAMP in mouse macrophages, LT-IIa(T14I) produced less than half the amount of cAMP in those cells [130]. In contrast, LT-IIa(T14D) produced no more cAMP in macrophages upon treatment with LT-IIa(T14D) than did macrophages treated with the fully non-toxic LT-IIa B pentamer [130]. These results suggested that immunomodulatory activities of LT-IIa required weaker binding to gangliosides than was required for toxicity.

Similar experiments were performed using LT-IIb(T13I). By ganglioside-dependent ELISA, LT-IIb(T13I0 had no affinity for GD1a, the ganglioside receptor which is strongly bound by LT-IIb, nor did it elevate cAMP levels in murine macrophages [129]. LT-IIb(T13I) however, retained the capacity to bind to splenic T cells, B cells, and macrophages, and exhibited full adjuvant capacity [129]. Thin-layer chromatographic (TLC) immunoblotting experiments revealed that binding affinity for GD1a was not completely ablated in LT-IIb(T13I). Rather, the mutant holotoxin bound weakly to GD1a and to several other minor gangliosides obtained from murine macrophages (T.D.C. and Hesham F. Nawar, unpubl.). Mice synthesize gangliosides which are decorated with either N-glycolylneurominic acids (NeuGc) or with Nacetylneurominic acids [22]. LT-IIb(T13I) bound preferentially to NeuGc-containing gangliosides in the TLC blots (T.D.C. and Hesham F. Nawar, unpubl.). Preferential binding to NeuGc gangliosides may confer the immunomodulatory activities to LT-IIb(T13I). A knock-out strain of mice in which the enzyme required to decorate gangliosides with NeuGc gangliosides, however, has recently been engineered. This KO mouse will facilitate

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experiments to evaluate the possibility that LT-IIb(T13I) utilizes NeuGc gangliosides as functional receptors for immunomodulation [131].

Functional interactions of Type II HLT with Toll-like receptor 2 (TLR2)

Recent reports indicate that the Type II HLT, unlike CT and LT-I, are not solely dependent upon binding to gangliosides for immunostimulatory activity. B pentamers of LT-IIa and LT-IIb (LT-IIaB and LT-IIbB, respectively), but not their respective holotoxins [132,133], functionally interact with TLR2 [125,132-134]. This effect is not observed with either CT, and likely will not be evident with LT-I. Both LT-IIaB and LT-IIbB, but not CT, induced TLR2-dependent nuclear factor κβ (NF-κβ) activation in a reporter cell [125,132-134]. Induction of IL-1β, IL-6, IL-8, and TNF-α by human THP-1 cells treated with LT-IIaB or LT-IIbB was inhibited by anti-TLR2 but not by anti-TLR4 antibodies [133]. Further evidence of the role of TLR2 in LT-IIbB-dependent immunomodulation was provided by mice having a defect in expression of TLR2. Peritoneal macrophages from $TLR2^{-/-}$ mice failed to respond to LT-IIaB or LT-IIbB [133]. Fluorescence resonance energy transfer (FRET) experiments demonstrated that LT-IIbB induces lipid raft recruitment of TLR2 and TIRAP, an adaptor protein, and that LT-IIbB, TLR2, and GD1a co-localize in the cell [133,134]. Interaction of LT-IIaB or LT-IIbB with TLR2 appeared to be partially mediated by binding of the B pentamer to gangliosides since LT-IIbB(T13I), a mutant which has altered GD1b binding activity, lacked the capacity to induce production of cytokines in THP-1 cells [134]. It is hypothesized that TLR2 agonist activity by LT-IIbB is elicited by a pentamer-induced formation of a multimolecular complex formed of the B pentamer, GD1a (or one of the other receptors for LT-IIb), and TLR2 [134] (Fig. 3). This interaction may be a functionally novel one. CD14, a usual coreceptor for TLR2 did not have a role as an accessory protein in LT-IIbB/TLR2-induced cell activation; CD14-deficient macrophages behaved in a manner similar to that observed in CD14-proficient macrophages when both were treated with LT-IIbB [134]. But what role does interaction of LT-IIbB with TLR2 have in immunomodulation? Ag-specific immune responses to a model Ag were augmented in mice intranasally immunized with LT-IIbB (T.D.C. and Hesham F. Nawar, unpubl). It will be interesting to determine if the mucosal and systemic adjuvant activities of LT-IIbB is a TLR2-dependent function. To address that issue, immunization experiments employing TLR2-deficient mice are ongoing.

EXPERT COMMENTARY

Many of the serious pathogens which cause disease in both industrial and developing countries invade and colonize individuals by infecting mucosal membranes. Thus, vaccines which stimulate protective immunity at the relevant mucosal site are desired. Unfortunately, vaccines which are administered parentally often fail to induce mucosal immunity. And, while perenteral vaccines have a reasonably good track record for safety, there is always a risk of infection and allergic responses when vaccines are administered by perenteral routes. Thus, there is a concerted effort to design safe mucosal vaccines. This goal, however, has been stymied by the failure of most vaccines when administered mucosally to induce strong and protective immune response, likely as a result of the toleragenic nature of mucosal tissues to foreign Ags. HLTs provide the means to augment the efficacy of mucosal vaccines. Yet, the inherent toxicities of the HLTs preclude their use in human mucosal vaccines. It is hoped that one or more mutants of CT, LT-I, LT-IIa, or LT-IIb will be engineered that will be non-toxic but will exert strong mucosal immune responses to foreign Ags. A non-toxic mutant enterotoxin which does not traffic along neuronal tracts would, of course, be most desirable.

It is also possible that research into the roles of gangliosides in HLT-dependent immunomodulation will provide data useful in developing other strong agonists for ganglioside-dependent immunomodulation. In that mode, the observation that the B pentamers

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of LT-IIa and LT-IIb interact functionally with TLR2 are especially intriguing. Toll-like receptors are potent regulators of both innate and adaptive immunity. Elucidating the mechanism(s) by which the B pentamers interact with TLR2 may reveal novel pathways and new agents for inducing innate and adaptive immune responses to useful immunogens.

FIVE-YEAR VIEW

Gangliosides are potent bioactive molecules which likely exert their effects by directly transducing signals or by aggregating transducing molecules upon crosslinking by ligands such as CT, LT-I, LT-IIa, and LT-IIb. Experiments employing mutant HLT with altered ganglioside binding activities have separated toxicity and adjuvanticity, thus making it more feasible to consider employing the mutant HLTs in human vaccines as agents to augment immune responses to the immunogen(s). The data are consistent with a model in which crosslinking of different gangliosides by CT, LT-I, LT-IIa, and LT-IIb or their respective mutant holotoxins and B pentamers stimulate distinctive immune responses. If so, mutant HLTs may be used in the future to direct immune responses into desired directions. New and exploitable mechanisms of immunostimulation may also be revealed by further investigating the properties of the B pentamers and holotoxins of wt and mutant LT-IIa and LT-IIb.

KEY ISSUES

1. Type I and Type II HLT are potent mucosal and systemic adjuvants which have the capacity to dramatically augment systemic and mucosal immune responses to co-administered Ags. Yet, the cellular and molecular processes which promote the immunomodulatory properties of HLTs have not been fully characterized or enumerated. These immunomodulatory properties are likely engendered by physical interactions of the HLTs with ganglioside receptors located on the surface of one or more types of immunocompetent cells.

2. Gangliosides belong to a large family of sugar-decorated lipids found usually on the plasma membrane of most eukaryotic cells including lymphocytes and lymphorenticular cells. Crosslinking of gangliosides has been shown to trigger signal transduction pathways that promote regulation of genes involved in immunomodulation. The Type I and Type II HLT have distinct binding properties for various gangliosides. Binding of an HLT to a particular ganglioside on the surface of an immunocompetent cell likely stimulates distinctive immune responses.

3. CT selectively inhibits IL-2 and IFN-γ from Th1 cells and increases IL-4 and IL-5 from Th2 cells. CT predominately induces production of IgG1, IgE, and secretory IgA in immunized animals. LT-I activates B cells and CD4+ T lymphocytes and induces apoptosis of CD8+ T cells. LT-I stimulated IL-2 synthesis, failed to stimulate production of IL-4, IL-5, and IL-12, and decreased production of IFN-γ by splenic cells. In combination with observations of the patterns of expression of co-stimulatory ligands, CT is thought to induce a Th2-type of immune response. In some cases, LT-I also induces a Th2-type of immune response. In other cases, however, a Th1-type of response is observed when LT-I is employed as an adjuvant.

4. The adjuvant properties of CT and LT-I are correlated with the ability of the holotoxins to induce cAMP in cells. These residual toxic activities preclude the use of the holotoxins as mucosal vaccines, particularly when administered via the intranasal route. HLTs likely traffic to the brain by retrograde transport along the olfactory nerves. Induction of proinflammatory cytokines and inflammatory

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histologies have been observed in the brain after immunization with CT and LT-I.

5. Non-toxic B pentamers of CT and LT-I have immunomodulatory properties. These properties, however, are less potent than those of their respective holotoxins. Use of the pentamers as mucosal adjuvants, however, may avoid the neurological problems associated with use of the holotoxins as adjuvants.

6. LT-IIa and LT-IIb, the Type II HLT, exhibit adjuvant properties equivalent to, but distinctive from those of CT and LT-I. The distinctive immunomodulatory properties of LT-IIa and LT-IIb are likely due to differences in their ganglioside binding patterns. Both LT-IIa and LT-IIb evoke a more balanced Th1/Th2-type of immune response. Immunization and in vitro experiments with LT-IIb demonstrate that strong binding to GM1 is not required for immunomodulatory activity.

7. The B pentamers of LT-IIa and LT-IIb, but not the CTB or LT-IB, interact functionally with TLR2 to activate NF-κβ, induce production of IL-1β, IL-6, IL-8 and TNF-α in human monocytic THP-1 cells. TLR2-dependent activity was not induced when cells were treated with either LT-IIa or LT-IIb holotoxins. FRET and fluorescent microscopic examination suggested that LT-IIbB stimulates formation of a multimolecular complex of pentamer, ganglioside, and TLR2 on the cell surface.

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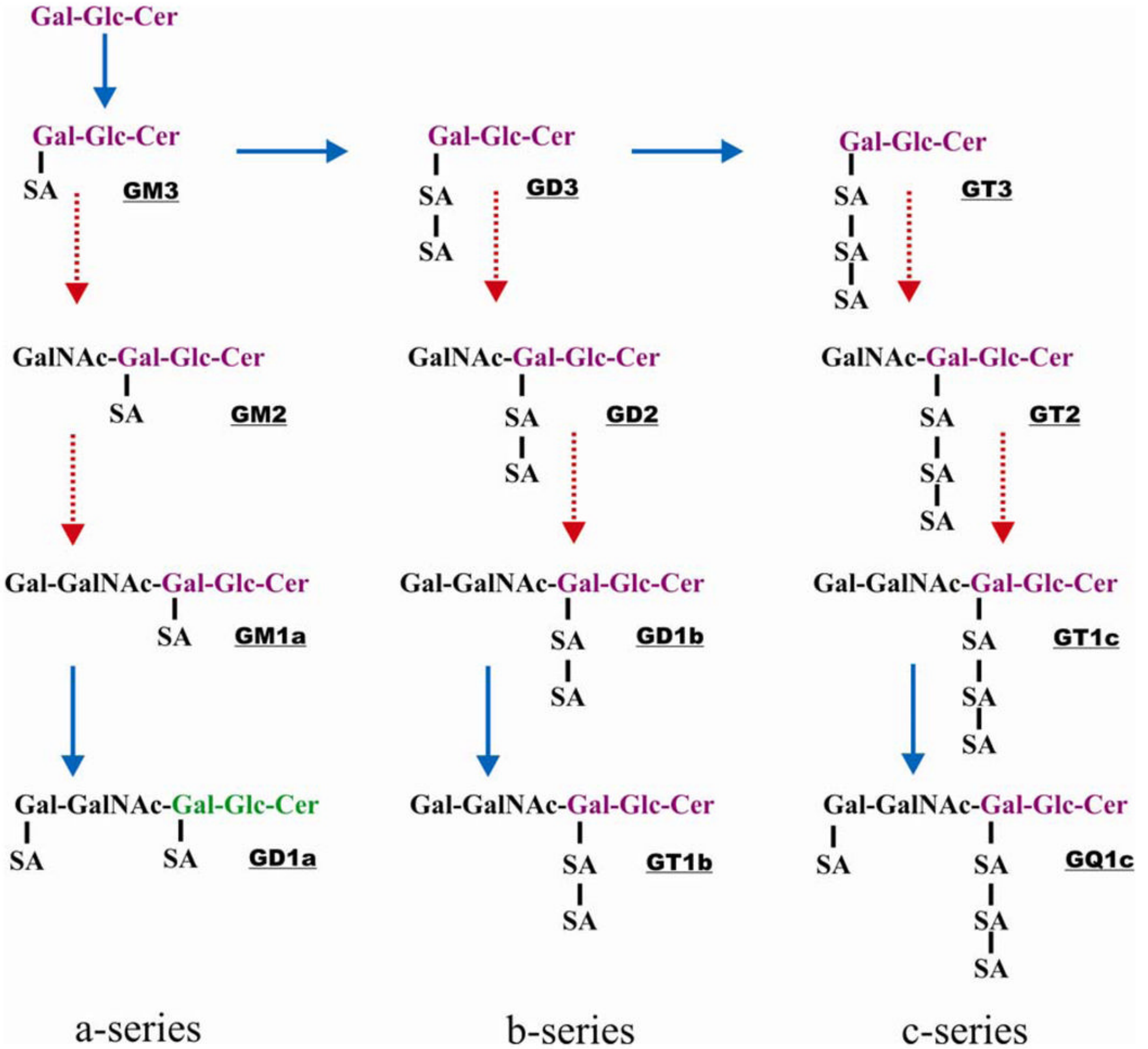

Fig. 1.

Pathways for the biosynthesis of the common a-, b-, and c-series of gangliosides. Synthesis of the various gangliosides involves the sequential activities of two types of enzymes: sialyltransferases (noted in solid lines) and glycosyltransferases (noted in dotted lines). Modified from - The Lipid Library. "Gangliosides: Structure, occurrence, biology and analysis". http://www.lipidlibrary.co.uk/Lipids/gang/. Copyright of W.W. Christie and/ where stated The Oily Press Ltd or others. Mr. Christie can be contacted by email at William.Christie@scri.ac.uk.

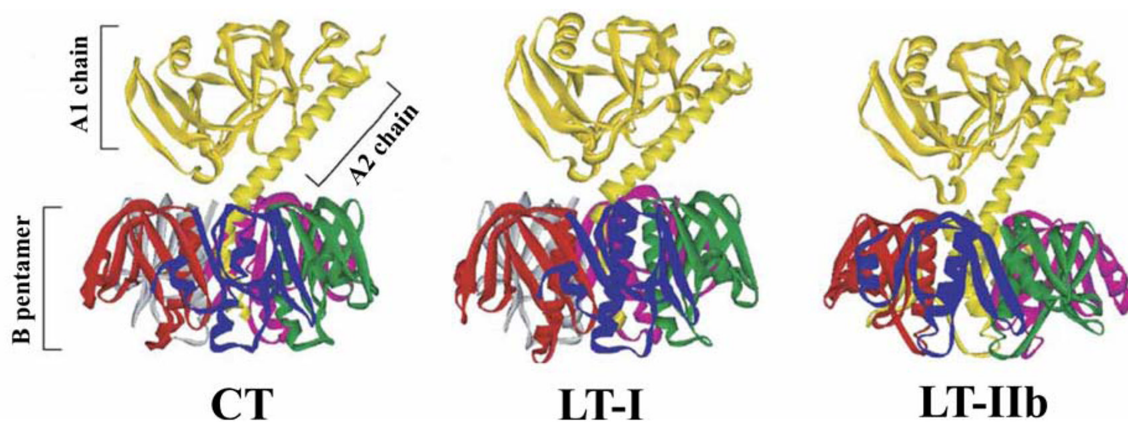

Fig. 2.
Ribbon diagrams of the crystal structures of CT [135], LT-I [135-137], and LT-IIb [138]. The A polypeptide, containing the catalytically-active A1 chain, is non-covalently bound to the B pentamer by interactions between the extended alpha helix of the A2 chain. A proteolytically-sensitive site is located in a site between the A1 and A2 chains bounded by two cysteines which form a disulfide bond. The five ganglioside binding pockets are located on the opposite side of the B pentamer in respect to the A polypeptide. Figure is modified from [139].

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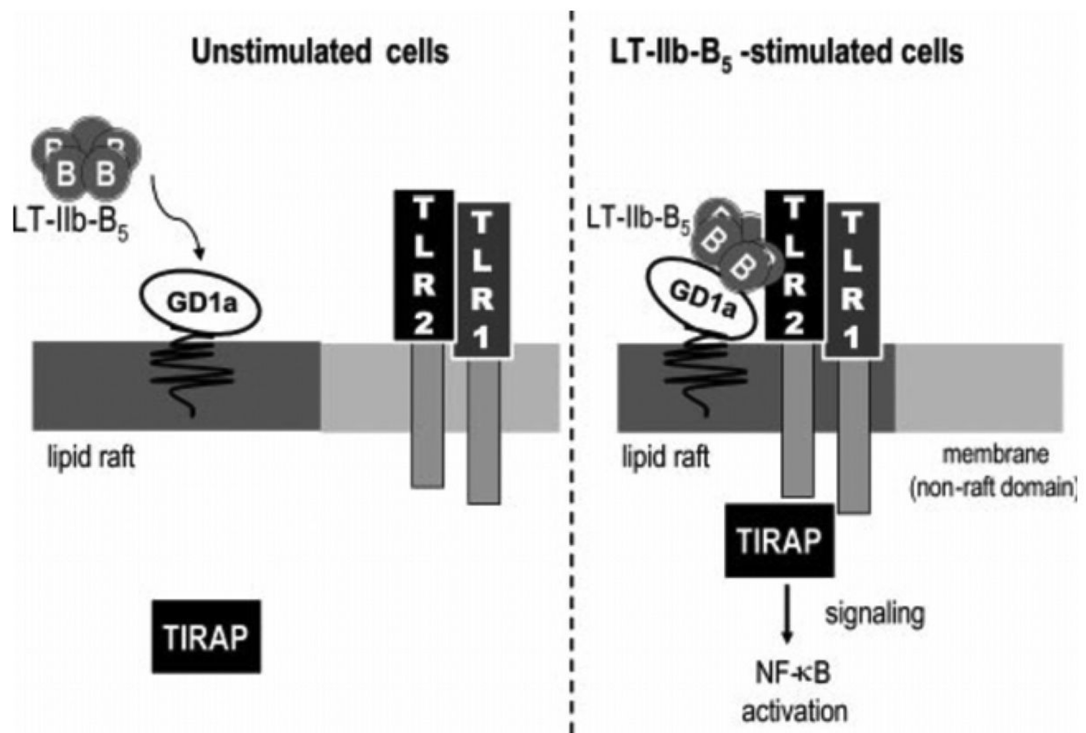

Fig. 3.

Model of the multimolecular complex formed by cooperative binding of LT-IIbB to GD1a and TLR2. After formation, the multimolecular complex is recruited into lipid rafts. Induction of TLR2 occurs on the cell surface and requires TIRAP, an adaptor protein, which co-localizes to the LT-IIbB/GD1a//TLR2 complex. Figure was originally published in [134].