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Mechanisms of Cholera Toxin in the Modulation of T_H17 Responses

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Abstract

Numerous studies have shown that T_H17 cells and their signature cytokine IL-17A are critical to host defense against various bacterial and fungal infections. The protective responses mediated by T_H17 cells and IL-17A include the recruitment of neutrophils, release of antimicrobial peptides and chemokines, and enhanced tight junction of epithelial cells. Due to the importance of T_H17 cells in infections, efforts have been made to develop T_H17-based vaccines. The goal of vaccination is to establish a protective immunological memory. Most currently approved vaccines are antibody-based and have limited protection against stereotypically different strains. Studies show that T-cell-based vaccines may overcome this limitation and protect hosts against infection of different strains.

Two main strategies are used to develop T_H17 vaccines: identification of T_H17-specific antigens and T_H17-skewing adjuvants. Studies have revealed that cholera toxin (CT) induces a potent Th17 response following vaccination. Antigen vaccination along with CT induces a robust T_H17 response, which is sometimes accompanied by T_H1 responses. Due to the toxicity of CT, it is hard to apply CT in a clinical setting. Thus, understanding how CT modulates T_H17 responses may lead to the development of successful T_H17-based vaccines.

Keywords

Interleukin-17; dendritic cells; T-cell differentiation; infection

I. INTRODUCTION

CD4⁺ T cells are central mediators of cellular immunity and have a crucial role in host defense. For many years, CD4⁺ T cells were classified as either T helper 1 (T_H1) or T_H2 cells by their effector cytokines and functions.^{1,2} T_H1 cells, which characteristically express interferon- γ (IFN- γ), are involved in the cellular immune responses and tissue

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inflammation, whereas T_H2 cells, expressing IL-4, IL-5, and IL-13 are responsible for the humoral immune responses and allergic diseases. However, this T_H1/T_H2 paradigm failed to explain some enigmas in immunity and autoimmunity.³ A regulatory T-cell subset is another CD4⁺ T lineage with immunosuppressive function, and its dysfunctions are associated with autoimmune diseases.⁴ In addition, the discovery of T_H17 cells revolutionized our concept of immunopathology and immune regulation.^{5,6} T_H17 cells produce proinflammatory cytokines,^{7,8} such as IL-17A, IL-17F, IL-22, IL-26, tumor necrosis factor- α (TNF- α), chemokine (C-C motif) ligand 20 (CCL20),⁹ and granulocyte macrophage colony-stimulating factor (GM-CSF).¹⁰ Although these cytokines all have proinflammatory features, they could act on different target cells and therefore contribute to different diseases.^{11–13}

T_H17 cells have been emphasized in the pathogenesis of autoimmune diseases and, in recent years, their physiological role in host defense has attracted significant attention.¹⁴ A protective role of IL-17A and T_H17 cells has been demonstrated in immunity against many bacteria^{15–20} and fungi.^{21,22} In murine models of airway infection, IL-17A is essential in host defense against extracellular bacterial pathogens, such as *Klebsiella pneumonia* and *Pseudomonas aeruginosa*, and against intracellular bacterial pathogens, such as *Chlamydia muridarum*, and *Mycoplasma pneumonia*.^{19,20,23}

Vaccination is one of the most effective ways to prevent infection, such as small pox, influenza, poliovirus, and hepatitis. Most of these vaccines induce neutralizing antibodies against pathogens; however, not all infectious diseases can be controlled by this antibody-based vaccination. The importance of T-cell responses in vaccinology is rapidly expanding.²³ Studies have shown that different T_H responses are required for particular pathogen clearance, and *vice versa*, pathogens also induce different T_H responses. Therefore, some bacterial components are used as adjuvants in vaccination to induce robust immune responses and even specific T_H-subset skewing responses. Cholera toxin (CT) from *Vibrio cholera* is one of the most well-studied bacterial toxins and is also used as an adjuvant in experimental animals. Recently, it has been reported to induce a T_H17-based response critical for host defense. However, its toxicity is a matter of serious concern in clinical application. Thus, studies enlightening the mechanism of the CT-mediated adjuvant effect will facilitate the development of effective T_H17-based vaccines.

In this review, we focus on T_H17 cell differentiation, the physiological roles of T_H17 cells in infection, the adjuvanticity potential of CT in promoting T_H17 responses, its mechanisms in the modulation of T_H17 cells, and finally, the potential CT derivatives in vaccine development.

II. CYTOKINES AND INNATE FACTORS IN T_H17 RESPONSES

Adaptive immunity is critical to inducing immunological memory against pathogens and is responsible for the long-term protective effect following vaccination. Innate immunity acts as the first-line defense to limit pathogen infection and spread. It is clear that innate immune cells also direct T_H differentiation through antigen (Ag) presentation and signals from direct cell contact and secreted cytokines.²⁴ Innate cells capable of processing and presenting

antigens to T cells are called professional antigen-presenting cells (APCs), which include dendritic cells (DCs), macrophages, and B cells. DCs are the major APCs to prime naïve T-cell differentiation. Immature DCs residing in tissues are highly capable of taking up Ag and presenting Ag on their surface. If maturation occurs, DCs undergo phenotypic changes, including the Ag presentation on their major histocompatibility complex II, expression of costimulatory molecules (CD40, CD80, CD86), and production of cytokines, chemokines, and chemokine receptors. The level of maturation determines the function of DCs.²⁵

When naïve T cells encounter antigen-primed DCs, DCs provide at least three known signals to trigger T-cell differentiation. The engagement of T-cell receptor (TCR, Signal 1) and co-stimulatory molecules CD40/80/86 (Signal 2) initiates naïve T-cell differentiation, and then cytokine/non-cytokine factors secreted by DCs or other cells (Signal 3) direct the differentiation to particular T_H subsets.²⁶ The transcription factors NF- κ B, nuclear factor of activated T cells (NFAT), and AP-1 are activated in naïve CD4⁺ T cells during initial stimulation (Signal 1+2), inducing specific gene transcription, such as IL-2, and leading cell proliferation and differentiation. In addition to initiating T-cell activation, the strength of TCR^{27–30} and the type of costimulatory molecules^{31–34} also determine T-cell lineage development. Cytokines expressed by DCs are the determining factors for T_H cell differentiation.³⁵ In general, T_H1 differentiation is mainly promoted by IL-12, IFN- γ , and IL-27. The T_H1 subset promotes cellular immune response and is required for host defense against intracellular bacteria and viruses. T_H1 cells secrete IFN- γ , IL-2, IL-10, and TNF α / β . IFN- γ and TNF α / β promote macrophage activation, nitric oxide production, and phagocytosis of pathogens. T_H1 cytokines also promote CD8 cytotoxic T-cell activation and proliferation to kill infected cells. T_H1 differentiation is inhibited by IL-4, which subsequently induces T_H2 differentiation. Conversely, T_H2 cell differentiation is inhibited by T_H1 cytokines, such as IFN- γ and IL-12. The T_H2 subset is mainly driven by IL-4 and is supported by IL-2, IL-7, or thymic stromal lymphopoietin (TSLP). The T_H2 subset plays an important role in humoral immunity and coordinates immune responses to large extracellular pathogens, like parasites. T_H2 cells secrete IL-4, IL-5, and IL-13, and these cytokines promote the IgE class switch of B cells as well as eosinophil and mast cell activation.

Although IL-17A production by CD4⁺ T cells was first described 20 years ago, T_H17 cells were not recognized as a distinct CD4⁺ cell subset until 2005.^{5,6} T_H17 cell differentiation has primarily been characterized in the murine system.⁸ Initially, IL-23, a member of IL-12, was suggested to promote T_H17 responses in experimental autoimmune encephalomyelitis (EAE). However, naïve murine T cells do not express the IL-23 receptor until activation, and now it is clear that IL-6 and TGF- β are the initial signals for T_H17 differentiation. In mice, IL-6 and TGF- β initiate T_H17 cell differentiation by activating Stat3 and inducing IL-23R and ROR γ t expression. IL-23 is then responsible for T_H17 cell maintenance and expansion.^{36–39} In addition, T_H17 cells also secrete IL-21, an autocrine signal, to sustain and promote their own differentiation via a Stat3-mediated manner.^{40,41} Later, IL-1 β was found to play an accessory role in murine Th17 cell differentiation, but recently it has been demonstrated to play a critical role in the early differentiation stages of murine T_H17 cells.⁴² IL-1R defective mice are resistant to the development of EAE; furthermore, IL1R^{-/-} mice failed to produce IL-23-induced IL-17A secretion. In humans, IL-1 β and IL-23 are the key determinates of T_H17 differentiation.

Many innate signals have been reported to promote T_H17 differentiation directly and indirectly. Activation of NLRP3 and caspase-1 in monocytes or DCs induces pro-IL-1 β and IL-18 production, and these cytokines promote IL-17A production from CD4 T cells and $\gamma\delta$ T cells.⁴³ Mice defective in NLRP3 or caspase-1 have significantly reduced T_H17 responses. The activation of the inflammasome can be triggered by a wide range of molecules, including pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). Cholera toxin (CT), *Escherichia coli* heat-labile enterotoxin, ATP, uric acid, amyloid- β , and the C3a complement have been reported to activate the inflammasome, subsequently leading to IL-1 secretion.⁴⁴

The gut microbiota has also been reported to play a central role in T_H17 differentiation in the intestine. For example, segmented filamentous bacteria are essential for gut T_H17 cell development.^{45,46} Polysaccharide A of *Bacteroides fragilis*, a commensal present in human, suppresses the IL-17A production from CD4 T cells. In addition, an increasing number of studies have shown that bacterial products, from either commensal or pathogenic bacteria,⁴⁷ such as lipopeptides and Toll-like receptor 2 (TLR2) ligands, could regulate T_H17 responses. TLR2 signaling indirectly promotes T_H17 differentiation via the induction of IL-1b, IL-23, and TGF- β from activated Langerhans cells,⁴⁸ and TLR2 signaling in CD4⁺ T cells also promotes T_H17 responses.⁴⁹

III. PHYSIOLOGICAL ROLES OF T_H17 CELLS IN INFECTION

T_H17 and regulatory T cells (Tregs) are most abundant in the intestinal lamina propria. T_H17 cells mainly secrete IL-17A, IL-17F, IL-22, and IL-26 and express CCR6. These cytokines induce the production of chemokines and antimicrobial peptides on epithelial cells and also induce neutrophilia, which contributes to mucosal immunity. In steady state, Treg cells are dominant and T_H17 cells secrete relatively low amounts of IL-17A to maintain the mucosal barrier. However, during infection, the suppressive functions of Treg cells are restrained and T_H17 cells secrete more IL-17A and other proinflammatory cytokines (e.g., IFN γ and TNF α) to protect against pathogens.

The functions of T_H17 cells and their cytokines in host defense have been demonstrated by loss-of-function or gain-of-function models of IL-17A, IL-17F, IL-22, and IL-23 and their receptors: IL-17RA, IL-17RC, and IL-23R. In murine models of airway infection, overexpression of IL-17A using a recombinant adenovirus enhanced bacterial clearance and survival after challenge with extracellular pathogen *K. pneumonia*. In support of this finding, the IL-17R-deficient mice were found to be more vulnerable to *K. pneumonia* infection.¹⁸ IL-17A has also been reported to play a protective role at other mucosal surfaces, with other types of pathogens, such as *Candida albicans* infection in the oral cavity and *Salmonella* dissemination in the intestines.^{22,50} In the colonic infection with *Citrobacter rodentium*, IL-17A, IL-17F, and IL-22 are involved in constraining the infection and the severity of gut pathology. Although the exact mechanism of T_H17 protection is not completely understood, the deficiency of IL-17 signal or other T_H17-associated cytokines in various infection models has shown that neutrophil recruitment is impaired in infected tissue, which is linked to the reduced expression of CXC chemokines.^{15–18}

IL-17A acts on immune and nonimmune cells to perform their functions in host defense.^{51–53} Our previous studies have found that IL-17A acts on airway epithelial cells to induce antimicrobial peptide human β -defensin 2, chemokine CCL20, and mucin MUC5B expression.^{54,55} In addition to airway epithelial cells, IL-17A also directly acts on airway smooth muscle (ASM)⁵⁶ and lung microvascular endothelial cells (LMVECs).^{57–59} IL-17RA and IL-17RC are expressed on the surface of LMVECs, and IL-17A significantly induces CXCL1 production in LMVECs. In skin immunity, IL-17A, IL-17F, and IL-22 also act synergistically on keratinocytes to induce the expression of β -defensin 2, S100A7, S100A8, and S100A9.⁶⁰ *In vitro* stimulation of intestinal epithelial cells with IL-17A, IL-17F, and IL-26 induces iNOS and lipocalin-2 expression and also increases the expression of CXCL-8 and CCL-20.^{61–64} IL-17A can also increase the expression of ICAM-1 in airway epithelial cells and claudin-1 and claudin-2 in intestinal epithelial cells, contributing to the tight junction formation in first-line barrier defense.^{65,66} Moreover, both IL-17A and IL-22 induce the expression of granulocyte colony-stimulating factor (G-CSF) and CXC chemokines.^{18,67,68} G-CSF is a major extracellular regulator of hemopoiesis, specifically for neutrophil differentiation. Both G-CSF and CXC chemokines contribute to the accumulation of neutrophils in the infected tissue and prevent pathogen dissemination.^{69,70}

IL-17 is also produced by CD8⁺ T cells⁷¹ and innate immune cells, such as $\gamma\delta$ T cells,^{72,73} iNKT cells,⁷⁴ and neutrophils,⁷⁵ and innate lymphoid cells (ILCs).^{76–78} Early IL-17A production by these innate cells provides an initial response to pathogens by recruiting neutrophils within 4–8 hours after infection. In the lung, $\gamma\delta$ T cells have been demonstrated to be the major source of early IL-17A production in response to some infections, such as *K. pneumonia*,⁷⁹ *M. tuberculosis*,^{73,80} and *Mycobacterium bovis*.⁸¹

IV. ADJUVANTICITY OF CHOLERA TOXIN (CT)

Cholera toxin (CT) and the closely related *Escherichia coli* heat-labile toxin (LT) are the most potent and studied mucosal adjuvants in experimental animals.^{25,82–84} Both CT and LT are ADP-ribosylating enterotoxins consisting of catalytic A and B subunits; the CT-B subunit can bind to the GM1 ganglioside receptor in all nucleated cells. CT-A subunit interacts with the host's G α subunit, resulting in the stimulation of host adenylate cyclase activity, which elevates the intracellular cAMP levels in various cells. However, CT causes severe diarrhea via intestinal epithelial cells, even at a low dose (10 μ g) of CT.^{85,86} Several studies have also indicated that CT and LT are associated with Bell's palsy (paralysis of the facial nerve).^{87–90} Because of this toxicity, CT is rarely used in human vaccines. Understanding the mechanism of CT-mediated adjuvanticity will help the development of safe and effective vaccine adjuvants.

Acting as an adjuvant, both the ADP-ribosyltransferase activity of CT and the binding ability of the B subunit to the GM1 receptor on target cells contribute to the induction of effective immune responses.^{91–93} The underlying mechanism of CT adjuvanticity potential has been explored but is incompletely understood. CT promotes innate immune responses by enhancing Ag presentation by antigen-presenting cells, like DCs and macrophages.⁹⁴ CT also induces IL-1 and IL-6 production by B cells, macrophages and DCs, and upregulates

the expression of costimulatory molecules (CD80, CD86, HLA-DR) on DCs.^{95–98} However, in some studies, CT reduced IL-12 production and induced IL-10 expression and the overall responses had an anti-inflammatory effect.^{99–101} Although *in vivo* CT administration in some studies induced a T_H1 response,^{102,103} in most studies CT promoted a strong T_H2-bias response to CT itself and to bystander antigens and suppressed a T_H1-bias response.^{97,104} The establishment of a T_H2-bias response is aided by the induction of T_H2-skewing DCs and may also be caused by the suppression of T_H1 cells. Moreover, CT also induces the expression of CXCR4 and CCR7 chemokine receptors on DCs; these cytokines enhance the migration of DCs to the secondary lymphoid tissue and promote the interaction between DCs and naïve T cells.⁹⁷

CT also induces B-cell activation and proliferation in an ADP-ribosylation-dependent pathway.¹⁰⁵ CT administration with Ag in mice has also significantly induced intestinal sIgA production and systemic IgG production via the oral, intranasal, or parenteral route.^{106–108} The ways in which CT induces mucosal IgA production has been addressed: CT enhances IL-4 production by T_H2 cells and IL-4 is required for induction of local IgA immunity.¹⁰⁶ CT also acts as a poor adjuvant in IL-4^{-/-} mice; however, the conjugation of CT with antigen, like OVA, can overcome the poor adjuvant effect. The administration of CT-OVA, but not the B subunit of CT (CTB)-OVA, can induce a strong mucosal anti-OVA IgA response in the gut lamina propria in both wild type and IL-4^{-/-} mice. This result suggests that the ADP-ribosyltransferase activity of CT is required for IgA production in an IL-4-independent pathway.¹⁰⁹ Thus, CT has been suggested to induce more humoral responses until the discovery of T_H17 cells.

V . CHOLERA TOXIN (CT) AND T_H17-BASED VACCINE

Currently, most approved vaccines are antibody-based, for example, small pox, hepatitis, and pneumococcal vaccines. However, not all pathogens can be eliminated by antibodies. The clearance of intracellular bacteria usually requires T-cell responses to kill the pathogens and infected cells. Also, the protective effect of antibody-based vaccine is limited to the vaccination strains. Therefore, the development of T-cell-based vaccines potentially provides comprehensive and serotype-independent protection employing both humoral and cellular immunity. As previously described, T_H17 cells are critical in the induction of protective immunity against many bacteria and fungal pathogens; thus, the idea to develop T_H17-based vaccine is emerging.²³

T_H17 cells have been shown to contribute the mucosal vaccine response against some pathogens.^{23,110} *Mycobacterium tuberculosis* (Mtb), the causative agent of tuberculosis (TB), has infected one-third of the world's population. Mice vaccinated with antigen from *Mycobacteria tuberculosis* (Mtb) provoke a T_H17 response and the CXCL-13 (B-cell chemoattractant) induction by IL-17A. This response is critical in protecting against Mtb infection.¹¹¹ *K. pneumonia* and *Pseudomonas aeruginosa*, opportunistic pathogens, cause pulmonary infection in immune-compromised patients. *K. pneumonia* is also a concern for the resistance to carbapenem antibiotics.¹¹² Immunization with heat-inactivated *K. pneumonia* in mice also induces T_H17-cell responses, whereas T_H17 cells are sufficient and required to protect from the infection of different serotype strains.¹¹³ The protection

involves the recruitment of neutrophils and antimicrobial proteins. A live-attenuated *P. aeruginosa* vaccine has been shown to induce a robust IL-17 responses, and the neutralization of IL-17A or mutation of IL-17R causes higher mortality in immunized mice. Also, IL-17A responses can protect mice from other serotype strains. Recently, several antigens of *P. aeruginosa*, such as PopB, have been shown to induce T_H17 responses.¹¹⁴ PopB-immunized mice were protected from lethal pneumonia in an IL-17-dependent manner. In addition, T_H17 responses are also required to control some fungal infections, such as *C. albicans*^{21,115,116} and *Aspergillus fumigatus*.¹¹⁷ A recent study shows that immunization of mice with attenuated fungi, *Coccidioides posadasii*, *Histoplasma capsulatum*, and *B. dermatitidis*, induces a robust T_H17 response and that this response is protective following fungal challenge. Similar to other studies, the protective mechanism was achieved through the recruitment and activation of neutrophils and macrophages to the alveolar space.¹¹⁸

In addition to Ag from pathogens, adjuvants are inorganic or organic additives to vaccines for targeting innate immunity to generate “danger signals.” Thus, the choice of adjuvant may generate different microenvironments to guide immune responses. The innate receptors and adjuvants involved in T_H17-based immunity have been summarized.¹¹⁰ Recent reports suggest that cholera toxin is a potent inducer for T_H17 responses in both mucosal and systemic vaccinations. The intranasal delivery of Ag with CT into mice induces a T_H17-dominated response, and this response is required for protection from bacterial infection, like *Bacillus anthracis* in mice.¹¹⁹ Datta *et al.* showed that DCs could become Th17-skewing DCs with secretions of IL-6 and other T_H17-polarizing factors after treatment with CT. Another report showed that the intranasal delivery of CT in mice induced T_H17-dominant responses and had a bystander effect on other antigens.¹²⁰ The CT-induced T_H17 response is abolished in IL-6 knockout mice. Interestingly, CT can also redirect T_H1- or T_H2-prone responses to T_H17-skewing responses. Recently, CT was used in the development of *Streptococcus pneumoniae*-specific T_H17 vaccine. The induction of IL-17A production and CD4⁺ T-cell activation are required for protection against *S. pneumoniae*.¹²¹ In the gut mucosa, T_H17 cells also play a role in vaccine-induced immunity against *Helicobacter* infections. Vaccination with *H. pylori* lysate or recombinant *H. pylori* urease along with CT in mice induces both T_H1 and T_H17 responses. The accumulation of T_H17 cells in the gastric mucosa can induce chemokines to recruit other inflammatory cells, like T_H1 cells and neutrophils for the pathogen clearance.^{122,123}

VI. MECHANISMS OF CT-INDUCED T_H17 RESPONSES

The adjuvant effect of CT is thought to involve the modulation of APCs. *In vitro* and *in vivo* studies demonstrate that CT induces T_H17-favoring cytokines (IL-1 and IL-6) from APCs. The binding of CT B subunit to GM1 receptor is required for its adjuvanticity potential to activate DCs, and CT also induced nuclear translocation of NF- κ B in DC in a GM1-dependent fashion.¹²⁴ However, whether NF- κ B signaling is a prerequisite for adjuvanticity is unclear. Lee *et al.* showed that the binding of GM1 to DCs is required for IL-6 production.¹²⁰ *In vitro*, CT treatment of bone-marrow-derived DCs (BMDCs) and intranasal delivery of CT into mice has also been shown to increase IL-6 production *in vitro* and in the lung. High levels of TGF- β detected in the lung mucosa, with IL-6 together, could contribute

to the induction of a T_H17 response. Both A and B subunits of CT contribute to T_H17 responses.^{119,125} Krishnamoorthy *et al.* found that the upregulation of c-Kit and its ligand, stem cell factor (SCF), on BMDCs and its downstream signaling via phosphatidylinositol-3 (PI3) kinase promote IL-6 secretion. The upregulation of c-Kit can be reversed by a cAMP antagonist, Rp-cAMPs. Rp-cAMP is known to effectively block the activity of cAMP downstream protein kinase A (PKA) but not EPAC (exchange protein activated by cAMP). Thus, the induction of IL-6 by CT in BMDCs is controlled via the cAMP-PKA-cKit-PI3K pathway. Datta *et al.* also reported this cAMP-dependent mechanism; they found that CT treatment on DCs induces the production of IL-1 β and β -calcitonin gene-related peptide via a cAMP-PKA dependent pathway. Neutralization of cytokines (IL-1 β , IL-6, IL-21, and TGF- β) in CT-treated DC culture media blocked IL-17A production from CD4⁺ T cells.

In addition to APC modulation, CT also directly regulates T-cell function. Several studies have shown that CT suppresses T-cell activation and proliferation.^{126–128} Specifically, T_H1 cells are more sensitive than T_H2 cells to inhibition by CT.^{129,130} However, in our study, CT upregulated IL-17A production by human CD4⁺ T cells and also promoted T_H17-cell differentiation.¹³¹ The addition of cAMP analogs mimicked the CT effect on IL-17A induction, thus suggesting the involvement of cAMP-signaling pathway in the regulation of IL-17A production. Utilizing different cAMP analogs and the promoter-reporter activity assay, we demonstrated that CT-induced IL-17A production occurs via a cAMP-PKA pathway. The cAMP-responsive element (CRE) in the IL-17A promoter is required for the transcriptional activation by CT. Our unpublished data indicate that the increase of phosphorylated CREB (cAMP response element-binding protein) and binding to CRE by CT is related to IL-17A promoter activity *in vitro*.

We also found that CT upregulated T_H17-promoting cytokines IL-6 and GM-CSF. GM-CSF is an activator for the development of DCs and has been used as a cytokine adjuvant in vaccine development.^{132,133} IL-23 has been reported to drive GM-CSF expression by T_H17 cells and then GM-CSF activates DCs to secrete more IL-23; this IL-23-GM-CSF circuit enhances T_H17 responses.^{10,134,135} Although the role of GM-CSF secreted from CT-treated T cells in T_H17 cell function *in vivo* needs to be clarified, the upregulation of GM-CSF may support DCs survival and activation; together with CT-mediated adjuvant effect on DCs, this upregulation will enforce T_H17 responses.

Because CT directly regulates IL-17A production via promoter activation and GM1 receptors are expressed in most nucleated cells, it is possible that CT may increase IL-17A production in other IL-17A-secreting cells.⁵³ Our unpublished data indicate that CT also induced more IL-17A production in CD8⁺ T cells (Tc17) via the cAMP-PKA signaling pathway. Tc17 is also involved in the pathogenesis of autoimmune diseases and host defense against infection. In CD4⁺ T-cell-depleted mice, Tc17 and other IL-17A-secreting cells compensated for the loss of IL-17A production from CD4⁺ T cells to protect the host against mucosal *Candida albicans* infection.¹¹⁵ Thus, the induction of Tc17 may also contribute to the adjuvant effect of CT. Whether CT also regulates other IL-17 producing innate cells, such as $\gamma\delta$ T cells, innate lymphoid cells, and iNKT cells, needs to be clarified. Early IL-17A production by these innate cells provides an initial response to pathogens, which confines the infection. Also, innate IL-17-producing cells may also enhance or direct the

development of later T_H17 responses.¹³⁶ Therefore, CT may both induce innate IL-17A responses in local tissues and generate T_H17 cells in the secondary lymphoid tissues.

VII. CHOLERA TOXIN, OTHER POTENTIAL MICROBIAL TARGETS IN T_H17 MUCOSAL VACCINE DEVELOPMENT

Mucosal vaccines have become a trend of vaccine development with many advantages and have been reviewed.^{137–139} Briefly, from an immunological perspective, successful mucosal vaccination induces both mucosal and systemic immune responses, and it effectively protects hosts against pathogens at the mucosal site of entry.¹³⁷ In medical application, the advantages of mucosal vaccines are lower cost, ease, needle-free administration, high patient compliance, and fast delivery during pandemic situations.¹³⁹ Microbial components are potential mucosal adjuvants due to their nature; they induce strong immune responses and they easily enter mucosal tissues. Emerging evidence shows that T_H17 responses are important and beneficial in mucosal immunity.¹⁴⁰ CT and LT are among the most potential mucosal T_H17 adjuvants; however, their toxicity caused a considerable occurrence of Bell's palsy in a clinical trial of influenza vaccine.^{141–143} The improvements of CT, LT, and other potential microbial targets in T_H17 vaccine development are listed in Table 1.

The B subunit of CT or LT alone has been co-administered with antigen to study their adjuvanticity potential. However, their potency was weaker than that of holotoxins and has the potential to induce immune tolerance. Also, CTB had less direct effects on T_H17 response in our study. Another way is to create cell-specific reception of holotoxins by conjugating A subunit to antigens or other cell-specific proteins, such as CTA1-DD linked to *Staphylococcus aureus* protein A and its derivatives, targeting B cells.^{25,144–146} Yet another approach is to reduce their toxicity by genetic modification, such as CT112, eCT6, LTK63, LTR72, LT196, and LT (R192G/L211A).^{147–152} The eCT6 mutant reduced toxicity by 10–20 fold and retained the adjuvanticity potential.¹⁵¹ LT (R192G/L211A) mutant is another prominent example of mucosal vaccination and induces T_H17 responses.¹⁴⁷

Some Toll-like receptors (TLRs)¹⁵³ and C-type lectin receptors (CLRs) have the potentials to induce mucosal T_H17 response.^{23,47,138} TLR2 signaling indirectly promotes T_H17 differentiation via the induction of IL-1 β , IL-23, TGF- β from activated Langerhans cells,⁴⁸ and TLR2 signaling in CD4⁺ T cells directly promotes T_H17 responses.⁴⁹ A TLR5 ligand, flagellin, has been shown to induce strong mucosal immunity and T_H17 responses by activating T_H17-skewing dendritic cells.^{154,156} Fungal components (α -mannan and β -glucan) bind to Dectin 1 and 2 as well as Mannose receptor, and their downstream signaling induces IL-1, -6, and -23 production as well as TNF production.^{157–162} These cytokines in turn promote the T_H17 response. In addition, microbial components from pathogens that induce T_H17 responses during infection can also be considered for development.

VIII. CONCLUSION

The elevation of cAMP by CT is essential to induce T_H17 responses. CT not only enhances T_H17 cell responses indirectly via dendritic cells but also directly promotes T_H17 and Tc17 responses, and thus CT can be applied in developing vaccines with Th17-skewing responses.

However, the toxicity of CT limits its therapeutic application in the clinical setting. At least two less toxic mutant forms of CT, CT112 and CTA1-DD, have been developed. Whether these mutants can also generate T_H17 responses in vaccination needs to be clarified. Another concern is the proinflammatory effect of T_H17 cells. The roles of T_H17 cells are well documented in autoimmune diseases. Thus, improper induction of T_H17 response in autoimmune patients may increase disease severity. Two subpopulations of T_H17 cells have been reported in EAE models, nonpathogenic and pathogenic T_H17 cells.¹⁶³ However, whether different T_H17 subsets contribute to the outcome of infection remains to be clarified. In addition, this T_H17 vaccination strategy may induce or worsen acute lung injury in lung infections. In one report, T_H17 responses induced by intranasal vaccination were detrimental in influenza infections because they induced strong lung neutrophilic responses.^{164,165} Thus, a more comprehensive understanding of Th17 cell differentiation, effector functions, and their roles in various infections is urgently needed to provide specific molecular targets that can ultimately be used to strengthen cell protective functions and constrain disease-specific functions.

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ABBREVIATIONS

DCs	dendritic cells
Tregs	regulatory T cells
CT	cholera toxin
Ag	antigen
APCs	antigen-presenting cells
EAE	experimental autoimmune encephalomyelitis

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TABLE 1 Current microbial targets in T_H17 adjuvant development

Adjuvants	Reference
Bacterial toxins	25, 144–152
CTA1-DD	
CT mutants (CT112, eCT6)	
LT mutants (LTK63, LTR72, LT196, LT (R192G/L211A))	
TLR ligands	153, 155
Monophosphoryl lipid A	
LPS	
Flagellin	
CLR ligands	157–160
α-mannan	
β-glucan	