

Recent developments in mucosal immunomodulatory adjuvants

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A large proportion of pathogens either invade through, or cause disease at mucosal surfaces. Many new generation mucosal vaccine candidates lack important immunostimulatory features of the original pathogens and thus often do not elicit sufficiently strong immune responses. Despite numerous efforts, there is a profound lack of available agents with mucosal immunomodulatory and adjuvant activity. Immunomodulatory adjuvants are often derived from pathogens and thus efficiently activate the innate immune system leading to subsequent development of strong, specific acquired immunity. In this review, recent advances in mucosal immunomodulators/adjuvants are described with special emphasis on recently developed detoxified cholera toxin and Escherichia coli heat labile enterotoxin derivatives, and the newly described Toll-like receptor ligands CpG DNA and imidazoquinoline compounds. These agents hold much promise as useful mucosal immunomodulators/adjuvants for induction of strong innate immune response and also for subsequent development of specific acquired immunity against mucosal pathogens.

Keywords Adjuvant, cholera toxin, imidazoquinoline, immunomodulator, immunostimulatory CpG oligodeoxynucleotide, mucosal immunity

Introduction

Over the last century, the development and widespread use of vaccines against a variety of pathogens has been a great triumph of medical science. Despite these successes, efficacious vaccines for many life-threatening pathogens and cancers are still not available. Traditional vaccines such as attenuated viruses, killed bacteria and bacterial toxoids often contain most of the features of pathogens required for generating danger signals to the immune system and are therefore sufficiently potent to elicit strong immune responses. In contrast, many new generation vaccines or vaccine candidates, particularly those based on recombinant protein or synthetic peptides, lack important immunostimulatory features of the original pathogens and thus often do not elicit sufficiently strong immune responses. Thus, there is a great need for useful immunomodulatory and/or immunodirecting adjuvants to render these vaccines efficiently immunogenic. Adjuvants are widely categorized into two different classes based upon their mode of action: vaccine delivery systems and immunomodulatory adjuvants. Vaccine delivery systems often have a particulate nature such as emulsions,

microparticles or liposomes and mainly facilitate targeting of enclosed antigens into the antigen presenting cells (APCs). Immunomodulatory adjuvants, on the other hand, such as lipopolysaccharide, cholera toxin (CT), or bacterial CpG DNA are principally derived from pathogens. These often represent pathogen-associated molecular patterns (PAMPs) recognized by so-called Toll-like receptors (TLRs) on host cells, including APCs. Recognition of PAMPs by TLRs triggers a signaling pathway that results in efficient activation of the innate immune system, leading to the subsequent development of strong, specific humoral and cell-mediated acquired immunity. Despite extensive assessments of a large number of candidate adjuvants, alum (aluminum-based mineral salts) is as yet the only worldwide-approved adjuvant for human use. Alum has a good track record of safety and has been considered the adjuvant of choice for vaccination against infections that can be prevented by systemic antibody response. Nonetheless, alum is known to be a poor adjuvant for induction of cell-mediated immune responses, especially cytotoxic T-lymphocyte (CTL) responses, required for combating many intracellular pathogens and cancers. Therefore, there is a particular need for new and potent adjuvants that can promote the induction and development of cell-mediated immune responses, including CTL responses. To date, no mucosal adjuvants have been approved for human use, therefore, there is an urgent need for the development of potent and safe mucosal adjuvants capable of eliciting strong cell-mediated, as well as humoral immune responses. The most widely used mucosal adjuvants in animals are CT and its close relative *Escherichia coli* heat labile enterotoxin (LT); however, both of these proteins are often considered to be too toxic for human use. Great effort has recently been made to define the possible extent of separating adjuvant activity from its toxic activity as a basis for the development of mucosal adjuvants effective for human use. The purpose of this review is to provide a brief overview of the recent advances in mucosal immunomodulators/adjuvants, with special emphasis on recently developed detoxified CT and LT derivatives and the recently described TLR ligands CpG DNA and imidazoquinoline compounds.

Bacterial toxins and derivatives

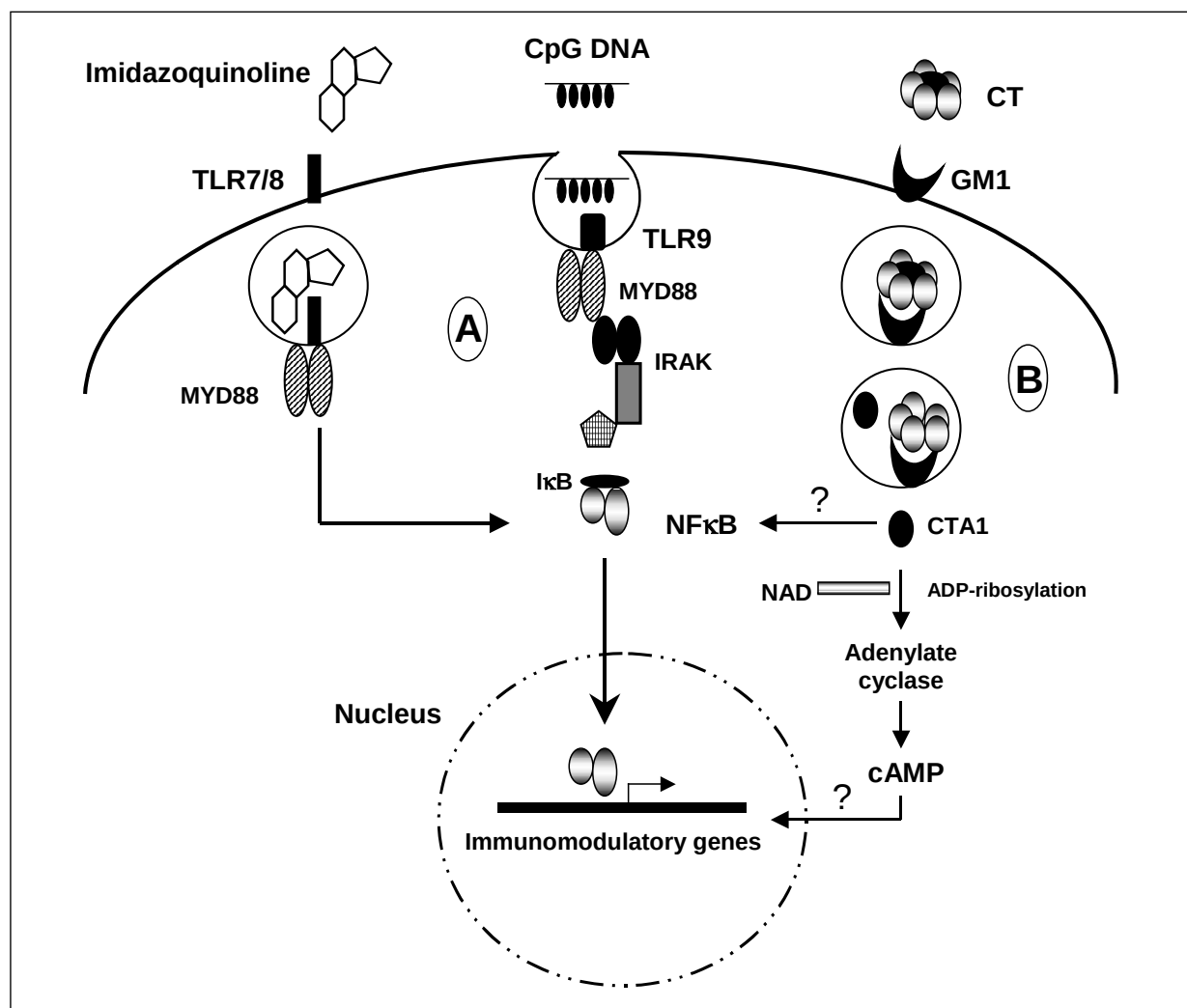
Both CT and LT have an analogous A-5B subunit protein structure. The B-subunit pentamer has a symmetric protein structure with a doughnut-like shape; the central pore in this structure holds CTA (or LTA) by non-covalent interactions. In the current model for cell intoxication by CT (or LT), and presumably also for their immunoadjuvant effect, the toxin binds to a cell surface receptor (GM1 ganglioside for CT and GM1 and additional galactose-containing glycolipids and glycoproteins for LT) and is endocytosed. The toxic active fragments CTA1 or LTA1 are then retrogradely transported to the cell cytosol. Once in the cytosol, CTA1 or LTA1 can enzymatically cleave NAD and attach the ADP-ribose moiety to the G α component of adenylate cyclase, thus causing an increase in the intracellular levels of cAMP. Some additional cell proteins with unknown functions are also ADP-ribosylated by CTA1 and LTA1. As mentioned, both CT and LT are powerful mucosal immunomodulators and adjuvants.

Mechanism of action

The mechanism of mucosal immunomodulatory and adjuvanticity of CT and LT involves multiple aspects of immune induction in the mucosa, which alone, or in combination, might explain their strong adjuvant action in stimulating mucosal IgA and other immune responses to admixed unrelated antigens after oral immunization (Figure 1). These effects include: (i) increased permeability of the intestinal epithelium leading to enhanced uptake of co-administered antigen; (ii) enhanced antigen presentation by a variety of cell types; (iii) promotion of isotype differentiation in B-cells leading to increased IgA formation; and (iv) complex stimulatory as well as inhibitory effects on T-cell proliferation and lymphokine production [1]. Among these many effects, those leading to enhanced antigen presentation by various APCs are probably of the greatest

importance for the potent adjuvant activity of CT and LT. CT increases antigen presentation by dendritic cells (DCs), macrophages and B-cells and has also been found, at least *in vitro*, to make intestinal epithelial cells become APCs. Consistently, CT has been demonstrated to upregulate the expression of the major histocompatibility complex/human leukocyte antigen-DR (MHC/HLA-DR) molecules, CD80/B7.1 and CD86/B7.2 co-stimulatory molecules, as well as chemokine receptors such as CCR7 and CXCR4 on both murine and human DCs and other APCs [2]. Furthermore, CT also induces the secretion of interleukin (IL)-1 β from DCs [3], thus supporting similar observations made for macrophages. IL-1 not only induces the maturation of DCs, but is also (by itself) an efficient mucosal adjuvant when co-administered with protein antigens [3] and may substantially contribute to the adjuvant activity of CT.

Figure 1. Mechanism of action of imidazoquinoline compounds, CpG DNA and CT on APCs.



Current knowledge of the mechanisms of action of the three potent mucosal immunomodulators/adjuvants: imidazoquinoline compounds, CpG DNA and CT on antigen presenting cells (APCs).

(A) The immunoactivating properties of the CpG DNA and imidazoquinoline compounds (eg, imiquimod and resiquimod) are elicited through recognition by TLR9 and TLR7/8, respectively, and subsequent activation of MYD88-dependent pathway leading to activation of NF κ B transcription factors in APCs.

(B) The immunomodulating activity of CT is at least partly linked to the upregulation of intracellular cAMP contents induced by the ADP-ribosylating activity of CTA1 on adenylate cyclase. It is tempting to speculate that the CTA1 subunit, either via induction of cAMP or through as yet unknown mechanisms, can activate immunomodulatory genes through NF κ B, thus leading to both activation and maturation of APCs.

CT has been suggested to primarily induce Th2-type immune responses characterized by CD4⁺ T-cells producing IL-4, IL-5, IL-6 and IL-10, and also by the production of IgA, IgG1 and IgE antibodies; LT, on the other hand, has been reported to induce a mixed Th1- and Th2-type immune response. However, our own work also indicates that CT induces mixed Th1- and Th2-type immune responses [3], in contrast to CTB, which appears to induce a more restricted Th2-type immune response. Thus, immunization of mice with DCs, which have been pulsed *in vitro* with a protein antigen (ovalbumin, OVA) linked to, or admixed with CT, did indeed induce both Th1 and CTL responses as well as a Th2-type immune response in an antigen-specific manner. In contrast, DCs pulsed *in vitro* with OVA linked to CTB gave rise to only a Th2-type immune response. The CT-induced stimulation of Th1 and CTL responses occurred even in the absence of the Th1-polarizing cytokines IL-12 or IL-18 as CT decreased production of these cytokines by DCs, and, furthermore, could not be linked to IL-1 β production as OVA-CTB and OVA-CT induced comparable levels of IL-1 β . Instead, and in contrast to OVA-CTB, OVA-CT markedly upregulated the production of RANTES and suppressed macrophage inflammatory protein (MIP)-1 α secretion, which may be of significance for its induction of Th1 and CTL responses together with a Th2 response. To avoid toxicity, isolated CTB and LTb have been explored as a way to augment immune responses against co-administered antigens, but their capacity as mucosal adjuvants has proved to be much less than that for the holotoxins. Indeed, both CTB and LTb are poor adjuvants, if at all active, in animals when given together with non-coupled antigens by the oral route. However, both CTB and LTb display a more significant adjuvant activity when given via the nasal route. Mice vaccinated intranasally with the influenza virus hemagglutinin (HA) vaccine PR8 H1N1 together with LTb had higher levels of antiviral IgA and IgG both in sera and in nasal and lung secretions compared with mice given the subunit vaccine alone, and were also protected against an intranasal viral challenge [4]. Adjuvanticity of CTB or LTb is much improved when antigens are coupled, possibly because of efficient presentation of the coupled antigen not only by DC and macrophages but also by naive B-cells [5].

Reduction of toxicity

Site-directed mutagenesis either at the junction between the A1 and A2 moieties or at the active site has permitted the generation of LT and CT mutant holotoxins that have reduced toxicity. In the first case, mutations are presumed to decrease the proteolytic release of the A1 enzymatic moiety [6], while in the second case, they would reduce the ability of the toxin to ADP-ribosylate its intracellular target [7]. In both instances, mutations lead to detoxification while still allowing for the expression of adjuvanticity. Significantly, however, adjuvanticity is not always retained and there are active-site mutants that have also lost their adjuvant capacity [8,9]. In a different approach to avoid CT toxicity, in particular GM1 binding activity of CT, a fusion protein designated CTA1-DD, comprising the enzymatically active CTA1 domain linked to an Ig (DD) binding moiety of *Staphylococcus aureus* protein, has been constructed. The CTA1-DD protein displays potent adjuvanticity when given systemically or intranasally, presumably because ADP-ribosylation is targeted to cells with surface Ig domains such

as B-lymphocytes; when given orally CTA1-DD shows no adjuvant activity, seemingly because it does not reach the relevant APCs. The latter deficiency could, however, be circumvented by combining this adjuvant with immune-stimulating complexes (ISCOMS), thereby also targeting DCs and/or macrophages in addition to B-cells [10•]. In a converse tactic, the CTA1 portion of CTA has been removed and antigens have been directly fused to CTA2 to produce A2/B chimeric mucosal immunogens. Such molecules may differentially enhance antibody responses and induce B7-dependent co-stimulation of CD4⁺ T-cells [11]. To achieve detoxification, yet another type of mutant was recently described in which peptides are added to the CTA1 amino end. In this new series the added peptides seem to reduce both enterotoxicity and ADP-ribosylating activity by steric interference with the CTA1 active site [12•]. In these constructs, adjuvanticity generally decreased together with enterotoxicity/ADP-ribosylation, however, a mutant (eCT6) that had 10- to 20-fold lower enterotoxicity displayed a level of adjuvanticity comparable to that of the wild-type CT. Moreover, another mutant with a longer peptide linked to CTA1 (eCT23) and no detectable toxic activity (although being much less potent in adjuvant activity than either CT or eCT6) was superior to CTB as both mucosal immunogen and as adjuvant for a co-administered antigen. Recently, a strategy has been employed in which the epidermal administration of plasmid vectors encoding CT or LT appears to improve the efficacy of co-administered DNA vaccines without causing overt toxicity. Both the CT and the LT vectors strongly augmented Th1-type cytokine responses (characterized by interferon (IFN)- γ) to multiple viral antigens when co-delivered with DNA vaccines. Furthermore, a Th2-type cytokine response (characterized by IL-4) was also elicited by both vectors [13].

CpG DNA and other Toll-like receptor ligands

The innate immune system senses infectious/harmful agents through pattern recognition receptors that specifically bind PAMPs, such as lipopolysaccharide, peptidoglycan, flagellin and bacterial CpG DNA. Recognition of PAMPs appears to be accomplished through a family of evolution-conserved, germline-encoded receptors, known as TLRs. Recognition of PAMPs by TLRs triggers a signaling pathway that leads to activation of nuclear factor- κ B (NF κ B) transcription factors and members of the MAP kinase family [14•]. This results in the production of pro-inflammatory cytokines and upregulation in expression of co-stimulatory molecules, thereby activating not only innate immunity, but ultimately also acquired immune responses. Thus, activation of the innate immune system through TLRs, by using TLR ligands, may represent an attractive strategy to enhance immune response against pathogens. In the following, we will describe how activation of innate immune response by using certain TLR ligands can impact mucosal immune responses.

Immunostimulatory CpG oligodeoxynucleotides

Bacterial DNA contains unmethylated CpG motifs, which distinguish bacterial DNA from vertebrate DNA. Vertebrate genomes uniformly contain a lower frequency of CpG motifs, and these are usually methylated [15]. Vertebrate immune systems appear to have evolved TLR9 that distinguishes bacterial DNA from self-DNA. Interactions

between unmethylated CpG motifs in bacterial DNA or in synthetic oligodeoxynucleotide (ODN) and TLR9 rapidly activates DCs, splenocytes, monocytes and macrophages through the Toll/IL-1-receptor signaling pathway to produce Th1-polarizing cytokines such as IFN γ , IL-1 β , tumor necrosis factor- α (TNF α) and IL-12 to upregulate co-stimulatory molecules on APCs and to activate B-cells for proliferation and IL-6 secretion (Figure 1) [16•,17•,18]. *In vivo*, systemic administration of CpG DNA was demonstrated to promote NK cell activity [19], to increase the total number of B-lymphocytes in the spleen [15], and to elevate plasma levels or tissue mRNA expression of IFN γ [20•], IL-12 [21] and TNF α [22]. Altogether, CpG DNA induces a predominantly Th1-type immune activation. The intracellular location of TLR9 and the fact that CpG DNA stimulates potent Th1-like immune responses suggest the possibility that immune recognition of CpG DNA may have evolved as a defense mechanism against intracellular pathogens. In animal models, systemic administration of immunostimulatory CpG ODN, in the absence of antigen, has been shown to induce non-specific Th1-like innate immune responses of a protective nature against infection with *Plasmodium yoelii*, *Listeria monocytogenes* and *Leishmania major* [23-25].

Despite the documented effects of CpG DNA on systemic innate and adaptive immunity, little is known about the role of CpG DNA in induction of innate immunity at mucosal surfaces. We have recently documented the impact of vaginal-mucosal administration of immunostimulatory CpG ODN on induction of innate immune responses in the murine female genital tract mucosa. Thus, a single mucosal-vaginal administration of immunostimulatory CpG ODN induces rapid production of the Th1-associated cytokines IFN γ , IL-12 and IL-18 in the female genital tract [26•]. Furthermore, such treatment stimulates rapid production of the CC chemokines RANTES [26•], MIP-1 α and MIP-1 β [AM Harandi and J Holmgren, unpublished data] in the murine female genital tract mucosa. The significance of the rapid CpG ODN-induced CC chemokine production in the genital tract mucosa is highlighted by the finding that infection of CD4 $^{+}$ T-cells by HIV-1 is antagonized by RANTES, MIP-1 α and MIP-1 β , the natural ligands of the HIV co-receptor CCR5 [27]. Remarkably, a single vaginal dose of CpG ODN, in the absence of any viral antigen, elicits protective immunity against genital herpes infection and disease [26•]. The protection led to the development of memory response conferring protection against re-infection. These data warrant further exploration for the use of immunostimulatory CpG ODN as a novel strategy to induce innate protective immunity against other sexually transmitted viral infections such as HIV infections. In our laboratory, we could also show that immunostimulatory CpG DNA elicits innate immunity in the gastrointestinal tract mucosa. Thus, intragastric administration of mice with CpG ODN was found to elicit local production of RANTES, MIP-1 α , MIP-1 β and the CXC chemokine IP-10 in the stomach and/or intestine. Importantly, intragastric administration of CpG ODN, in the absence of any exogenous bacterial antigen, suppressed bacterial colonization in the stomach of mice with an already established *Helicobacter pylori* infection [28]. In addition to the effect of CpG DNA on the induction of innate immune response, several features of the immune stimulation induced

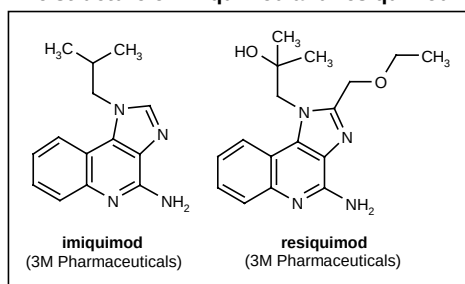
by CpG DNA, including the generation of a Th1-like cytokine milieu, anti-apoptotic activity on B-cells and DCs, and upregulation of co-stimulatory molecule expression on APCs suggest potential efficacy of CpG DNA as a vaccine adjuvant. The utility of CpG DNA as a potent Th1-like adjuvant for mucosal vaccination of primate and non-primate animals with various antigens, including hepatitis B surface antigen (intranasally and orally), whole killed influenza virus (intranasally or orally) and MAP-M2, a synthetic measles virus peptide (intranasally), envelope glycoprotein B from HSV-1 (intranasally), tetanus toxoid (intranasally, intrarectally and orally) and *Mycobacterium bovis* BCG (intranasally) has been demonstrated [29,30]. Importantly, some of these studies confirmed that CpG DNA is a stronger Th1-like adjuvant for inducing B-cell and T-cell responses than complete Freund's adjuvant, while the toxicity of CpG DNA was shown to be far less [31]. It has also been reported that CpG covalently conjugated to protein antigen exhibits novel joint properties as promoters of antigen uptake and DC activation, thereby potentiating the ability of DCs to generate Th1-mediated immune response [32]. To date, the efficacy of different immunostimulatory CpG DNA to combat various infectious diseases and cancers are under preclinical or clinical evaluation. Preliminary data from early-stage clinical trials of CpG DNA as an adjuvant with hepatitis B surface antigen, as an anti-allergic agent and as immunotherapy for cancers (ie, lymphoma, melanoma and basal cell carcinoma) appear to be promising [33].

Imidazoquinoline compounds

Imiquimod and its derivative resiquimod (R-848; 3M Pharmaceuticals) are members of a new group of low molecular weight imidazoquinolinamines that activate immune cells (Figure 2). The immunoactivating properties of these pharmacological agents are elicited through recognition by TLR7 and/or TLR8, and subsequent activation of the MYD88-dependent pathway, leading to activation of NF κ B transcription factors (Figure 1) [34•,35]. *In vitro* studies using non-human or human monocytes have established the potent effects of imiquimod and resiquimod in the induction of a variety of cytokines, such as IFN α , TNF α , IL-1 β , IL-6, IL-12, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte CSF (G-CSF) and MIP-1 α [34•,36,37]. Moreover, Langerhans cells exhibit functional activation and enhanced induction of T-cell proliferation in response to imiquimod or resiquimod. *In vivo* studies of humans and animals treated orally or topically with imiquimod or resiquimod have confirmed the induction of IFN α and TNF α production [36]. Vaginal-mucosal administration of imiquimod shows promise in the treatment of genital herpes. Thus, imiquimod given intravaginally reduced primary lesions, reduced virus shedding and reduced the virus content in spinal cords from herpes simplex virus (HSV)-infected guinea pigs [38]. Imiquimod has also been successfully used for the treatment of genital human papillomavirus infection and is now an approved treatment for external genital warts. Mechanism of action studies indicate that the therapeutic properties of imiquimod and resiquimod are related to the ability to generate pro-inflammatory cytokines and Th1-type immunity [37]. Thus, topical administration of TLR7/TLR8 ligand immunostimulatory imiquimod or resiquimod represents a new approach in the therapeutic treatment

paradigm for the treatment of mucocutaneous viral diseases at their site of infection. In addition to the stimulatory effects of imiquimod on innate immune response, the adjuvant properties of imiquimod and resiquimod for enhancing efficacy of vaccines have also been established in animal models [37]. Resiquimod showed potent activity as a vaccine adjuvant in a mouse model, inducing high serum concentrations of TNF α , IL-12 and IFN γ . Imiquimod administered in combination with HSV-2 glycoproteins enhanced the effectiveness of HSV-2 immunization, especially in reducing recurrent genital herpes disease [39].

Figure 2. The structure of imiquimod and resiquimod.



Conclusions

The last decade has brought much progress in the development of detoxified CT and LT, providing a new generation of CT and LT derivatives that display strong adjuvant activity with little or no toxicity. Based on the rapid pace of investigation in this field, it seems likely that the future holds much promise for the detoxified CT and LT derivatives in the realm of mucosal adjuvants for human use. The discovery of PAMPs as potential ligands for TLRs has led to revolutionary progress in the field of immunomodulators/adjuvants. Among the PAMPs, the bacterial immunostimulatory CpG DNA TLR9 ligand shows especially promising potent immunostimulatory and/or adjuvant activity at mucosal surfaces. Recently, non-PAMP TLR7/8 ligand imidazoquinoline compounds have been identified as strong immunostimulator/adjuvants at genital tract mucosa. Based on this, it seems likely that future investigation of both PAMP and non-PAMP TLR ligands will continue to be a fruitful area of research for development of novel mucosal immunomodulators/adjuvants.

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