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TLR-Based Immune Adjuvants

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Abstract

This work describes the nature and strength of the immune response induced by various Toll-like receptor ligands and their ability to act as vaccine adjuvants. It reviews the various ligands capable of triggering individual TLRs, and then focuses on the efficacy and safety of those agents for which clinical results are available.

Introduction

The innate immune system provides the host with a rapid mechanism for detecting infection by viral, microbial and fungal pathogens. Cells of the innate immune system express pattern-recognition receptors, including Toll-like receptors, that bind conserved molecules expressed by a wide variety of infectious agents. Triggering via TLR stimulates the production of pro-inflammatory cytokines/chemokines and type I IFNs that increase the host's ability to eliminate the pathogen [1-4]. This innate immune response also supports the subsequent development of adaptive immunity, and thus can be harnessed to accelerate and enhance the induction of vaccine-specific responses [5]. This review examines the types of response elicited by various TLR ligands, focusing on the efficacy and safety of those agents currently in clinical trial.

TLR 2 (and associated TLR dimers)

General overview

TLR2 interacts with a broad and structurally diverse range of ligands, including molecules expressed by microbes and fungi. Multiple TLR2 agonists have been identified, including natural and synthetic lipopeptides (e.g. *Mycoplasma fermentas* macrophage-activating lipopeptide (MALP-2)), peptidoglycans (PG such as those from *S. aureus*), lipopolysaccharides from various bacterial strains (LPS), polysaccharides (e.g. zymosan), glycosylphosphatidyl-inositol-anchored structures from gram positive bacteria (e.g. lipoteichoic acid (LTA) and lipo-arabinomannan from mycobacteria and lipomannas from *M. tuberculosis*) [6]. Certain viral determinants may also trigger via TLR2 [7]. Yet uncertainty exists concerning the mechanism(s) by which TLR2 recognizes such a wide array of ligands, leading some to suggest that contamination with lipopeptides (which trigger TLR2 at picomolar levels [6]) may underlie some of the reported activity. In this context, several groups report that highly purified/synthetic peptidogylcans are unable to trigger via TLR2 (in contrast to previous claims) yet retain their ability to stimulate via Nod1/2 [8-10]. In recognition of these concerns, this review will focus on the use of lipopeptides as vaccine

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adjuvants, as available data suggest they represent the most relevant of the TLR2 agonists being evaluated for that purpose.

TLR2 ligands

Since TLR2 is expressed on many different cell types (including dendritic cells, macrophages and lymphocytes) the mechanisms by which bacterial lipopeptides manifest their adjuvant properties are diverse. Preclinical testing indicates that lipopeptides co-administered with or physically linked to Ag can i) induce DC maturation leading to the up-regulation of co-stimulatory signals and Ag-presenting molecules (e.g. MHC class II, CD80, CD83, IFNg, IL-12) [11-13], ii) stimulate macrophages to release cytokines (e.g. TNF, IL-1, IL-6) [14;15], iii) promote the maturation and activation of B cells leading to increased production of Ag-specific IgG and IgM Abs [16;17] and iv) boost the generation of antigen specific $CD8^+$ T cell (CTL) responses [18-20].

Two strategies are commonly utilized to generate TLR2-dependant lipopeptide vaccines: i) conjugating bacterial lipopetides or their synthetic analogues to peptide and ii) covalently linking palmitic acid to peptide antigens. Bacterial lipopeptides are structural components of cell walls. They consist of an acylated s-glycerylcysteine moiety to which a peptide can be conjugated via the cysteine residue. The bacterial lipopeptides most frequently used as vaccine adjuvants are MALP-2 and it's synthetic analogue di-palmitoyl-S-glyceryl cysteine (Pam_2Cys) or tri-palmitoyl-S-glyceryl cysteine (Pam_3Cys).

The alternative approach to generating TLR2-dependant lipopeptide vaccines involves modifying the antigenic peptide with N^{ε}-palmitoyl-lysine [21]. Pre-clinical studies of these lipidated Ag constructs show that they i) induce the maturation of DCs, increasing the production of pro-inflammatory cytokines (e.g. IL-12, TNFα, IFNg) [21;22], ii) activate B cells to increase production of IgG Abs [21;23;24] and iii) enhance the generation of Ag specific CTL responses [21;25-28]. Thus, preclinical data support the conclusion that the immunogenicity of peptide-based vaccines is significantly improved by conjugating them to either bacterial lipopetides or palmitic acid moieties.

Clinical activity

Multiple TLR2 ligands have undergone clinical testing (see Table 1). The most extensively studied was Pam_3Cys linked to outer surface protein A (OspA) of *B. burgdorferi* (the spirochete that causes Lyme disease). This Lyme disease vaccine (LYMErix™) was tested in over 20,000 volunteers [29;30]. The induction of protective immunity correlated with the development of Abs against an epitope on the C-terminus of OspA (protective IgG titer >1,400 EIA units/ml). Three doses of the vaccine induced protective immunity in >75% of subjects, and was licensed by the FDA in 1998 for general use. The manufacturer voluntarily withdrew this product 3 years later amidst media coverage of possible autoimmune side effects which led to a decline in sales [31;32]. Of note, neither the FDA nor the CDC found a connection between the vaccine and the development of autoimmunity [31].

Pam_3Cys was also tested as an adjuvant in combination with a peptide vaccine targeting malaria. The vaccine contained multiple B cell epitopes plus a universal T cell epitope derived from the *Plasmodium falciparum* circumsporozoite protein (CSP) [33]. Following three immunizations, all volunteers (N = 10) developed detectable levels of peptide specific IgG Abs with titers ranging from 160 to 20,240 (2,750 GMT). The addition of Pam_3Cys resulted in the production of epitope-specific IgG1, IgG3 and IgG4 Abs, while an earlier study using alum and QS21 as adjuvants induced only IgG1 and IgG3 isotypes [34]. The

peptide-specific IgG titers and the CSP responses induced by the vaccine formulated with Pam_3Cys or alum/QS21 were similar.

A palmitic acid conjugated vaccine was recently tested in subjects with chronic Hepatitis B infection. That vaccine (Theradigm-HBV) consisted of a CTL peptide from the HBV core antigen plus a helper T lymphocyte (HTL) peptide which was palmitoylated at the N-terminus [35]. The addition of palmitic acid significantly improved the immunogenicity of the CTL and HTL epitopes which were otherwise poorly immunogenic. In a phase I trial involving 26 healthy volunteers, four doses of this vaccine induced a dose-dependent HBV-specific CTL response that persisted for more than 9 months [36]. However CTL responses were not induced in patients chronically infected with HBV [37].

Vaccines targeting HIV using palmitic acid extended peptide antigens were also evaluated in phase I and II trials [38]. Those vaccines contained a mixture of up to six different HIV Ags (including Nef, Gag and Env) that were modified to express a single palmitoyl chain at their C terminus to serve as an endogenous adjuvant. After 4 immunizations, 93% of volunteers generated IgG Abs and 86% showed a specific CTL response (Ag alone had no effect). Both responses persisted for at least 2 years in a majority of participants, indicating that long-lasting memory was generated [39]. Unfortunately, this vaccine failed to boost HIV-specific CTL responses in HIV infected subjects (N = 43) [40].

The TLR2 ligands described above are easily incorporated during peptide synthesis, yielding a well-defined and totally synthetic vaccine. These lipid adjuvants consistently boosted both humoral and cell mediated responses to peptide-based vaccines in normal healthy volunteers. The magnitude and duration of the Ab responses suggest that they would be sufficient to prevent many infectious diseases. However the ability of such vaccines to promote CTL responses was less impressive, nor were they effective in post-exposure settings. Thus, their utility for the prevention/treatment of chronic infectious diseases (such as HPV or HIV) is uncertain.

Safety

Clinical trial results indicate that lipopetide vaccines are generally safe. The LYMErix™ vaccine was well tolerated in clinical trials involving over 20,000 individuals followed for up to 6 years. Local adverse events, in particular redness and/or swelling at the injection site, were mild to moderate and generally lasted only 2-3 days. Less than 4% of the vaccinees reported systemic complaints, primarily myalgia or fever, and no hypersensitivity reactions or abnormal laboratory results were observed [29;30;41]. By 2001, over 1.4 million doses of LYMErix™ had been administered in the United States. The Vaccine Adverse Events Reporting System database included 905 reports of mild self-limiting reactions and 59 reports of arthritis associated with vaccination [42].

The safety of LYMErix™ differed somewhat from the effect of lipopetide vaccines used in anti-HIV trials. A meta-analysis of 10 such trials involving a total of 200 healthy volunteers and 48 HIV infected subjects showed that 18 individuals (including a number of normals) experienced grade 3 systemic events related to vaccination, including asthenia, fever, headache and arthralgia [38], a reactogenicity profile that raises concern about the safety of this form of adjvuant.

TLR3

General overview

TLR3 is expressed within the endosomal compartment of conventional dendritic cells and macrophages and is present on the surface membrane of non-immune cells including

epithelial cells [2]. TLR3 is triggered by double-stranded RNA (dsRNA) produced during the replication of most viruses. The interaction of TLR3 with dsRNA initiates a TRIF-dependent signaling cascade that progresses through the activation of NF-kB, MAP kinases and IRF3 and culminates in the production of inflammatory cytokines and type I IFNs [43;44]. Thus, TLR3 is distinct from all other TLRs in that it does not utilize the MyD88-dependent pathway for signaling.

A potential advantage of TLR3 agonists is that triggering via this signaling cascade facilitates Ag cross-presentation, in which $CD8^+$ T cells are primed by exogenous Ag presented in the context of MHC class I molecules, improving the generation of cytotoxic T cells [45;46]. Cross-presentation is also enhanced by the type I IFNs induced following TLR3 stimulation [47]. Thus, TLR3 ligands are considered excellent candidates as adjuvants for vaccines targeting the induction of a strong cellular immune response [48].

TLR3 ligands and their immunogenicity

Poly I:C (polyriboinosinic:polyribocytidylic acid) is a synthetic analog of dsRNA and the archetypal TLR3 ligand [49]. Since poly I:C interacts with additional receptors (including retinoic acid-inducible gene I, melanoma differentiation-associated gene 5 and double-stranded RNA-dependent protein kinase), it's adjuvanticity cannot be uniquely ascribed to TLR3 activation [50;51]. In a murine model of influenza virus infection, intranasal administration of poly I:C with an HA-based influenza vaccine induced a strong IgA anti-HA response in the nasal mucosa and IgG response in serum, whereas vaccination without poly I:C had little effect. The addition of poly I:C protected mice from lethal nasal or pulmonary viral challenge [52]. Other studies in murine models show that poly I:C can enhance the efficacy of peptide-based cancer vaccines by promoting tumor specific T cell responses [53-56].

Despite promising findings in mice, poly I:C had limited success inducing IFNs or mediating anti-tumor activity in primates (including human) due to degradation by serum nucleases [57;58]. Higher doses of poly I:C caused severe safety problems, including shock, renal failure and coagulopathies in phase I-II clinical trials of cancer patients [59]. To improve the activity and safety of TLR3 ligands, derivatives of poly I:C were produced, of which poly ICLC and poly I:C_{12}U (also known as Ampligen®) are the most widely studied. Although clinical trials involving these adjuvants have been initiated, no published data on their activity and safety is available.

Poly ICLC is a synthetic double-stranded polyriboinosinic-polyribocytidylic acid stabilized with poly-L-lysine carboxymethyl cellulose. It is 5 -10 times more resistant to hydrolysis by primate serum than poly I:C [60]. In a murine CNS tumor model, poly ICLC improved IFNg production, enhanced CTL responses by 2-4 fold and improved survival 2-fold vs vaccine alone [61]. A Study of human papillomavirus (HPV) infection in rhesus macaques showed that administering HPV antigen plus poly ICLC increased IFNg secreting cell numbers by 2-fold and enhanced HPV-specific CD4+ T cell responses by 5-fold when compared to vaccine alone [62]. Inclusion of poly ICLC boosted the titer of HPV-binding Ab by up to 1,000-fold.

Synthetically modifying poly I:C to introduce mismatched bases (uracil and guanine) generates TLR3 ligands whose immunostimulatory potential is similar to poly I:C, but with less toxicity [63]. Poly I:C_{12}U is a representative example of such synthetic mismatched dsRNA. In pre-clinical studies, poly I:C_{12}U stimulated human monocyte-derived DC to mature and produce IL-12 (and decrease their production of IL-10) [64]. These activated DC promoted antigen-specific CTL responses and the differentiation of CD4+ T cells toward a Th1 phenotype [65]. In an influenza virus H5N1 infection model, intranasal administration

of H5N1 vaccine with poly I:C_{12}U increased antigen-specific IgA production by nearly 3-fold [66]. Viral titers following challenge were significantly reduced, and nearly all mice were rescued from lethal H5N1 virus infection.

Safety

Initial clinical studies involving poly ICLC showed that high dose therapy was associated with mild to severe side effects including hypotension, fever, and anemia. Once the dose and frequency of delivery were optimized, this TLR3 agonist was well tolerated in humans [67-74]. Several phase I-II clinical trials evaluating the activity and safety of poly ICLC as a cancer vaccine adjuvant are ongoing.

Poly I:C_{12}U was well-tolerated in several long-term phase I-II clinical trials involving cancer patients [75]. This reduced toxicity when compared to poly I:C may reflect the accelerated hydrolysis and shorter plasma half-life of poly I:C_{12}U [75;76]. Available data suggest that this agent is the most promising TLR3 ligand under clinical development. Studies of poly I:C_{12}U as monotherapy against viral infection and phase II and III clinical trials examining their use in HIV and chronic fatigue syndrome are underway [77;78]. Use of this TLR3 ligand as a vaccine adjuvant in humans is less advanced, with a phase I clinical trial in patients with ovarian cancer scheduled to begin in the near future [65].

TLR4

General overview

TLR4 is expressed by cells of the innate immune system, including conventional dendritic cells and macrophages. It is also expressed by many non-immune cells including fibroblasts and epithelial cells [2;79;80]. Triggering via TLR4 induces a signaling cascade that utilizes both the MyD88- and TRIF-dependent pathways, leading to NF-kB and IRF3/7 activation, respectively [2;81]. Among TLRs, only TLR3 and TLR4 stimulate the production of type I IFNs via TRIF [2;81]. TLR4 activation typically induces robust IL-12p70 production and strongly enhances Th1 type cellular and humoral immune responses [82;83].

TLR4 ligands

A diversity of ligands reportedly interact with TLR4, including lipopolysaccharides (LPS), mannans (Candida albicans), glycoinositolphospholipids (Trypanosoma), viral envelope proteins (RSV and MMTV) and endogenous antigens including fibrinogen and heat-shock proteins [2;81]. LPS, which is found in the outer membrane of gram negative bacteria, is the most widely studied of the TLR4 ligands, and virtually all clinical trials involving TLR4 adjuvants examine derivatives of LPS. LPS is a complex molecule, and it is the lipid A portion composed of polyacylated diglucosamine lipids that mediates interactions with TLR4 [84;85]. Although the immunostimulatory capacity of LPS has been known for decades, the intact molecule is highly toxic, preventing it's use as a vaccine adjuvant [86]. Fortunately, the monophosphoryl lipid A (MPLA) component of LPS (purified from the cell wall of *Salmonella minnesota* R595 and detoxified by mild hydrolytic treatment) is considerably less toxic yet maintains immunostimulatory activity [87-89]. Studies in rabbits, guinea pigs, dogs and horses show that MPLA is 1,000-fold less toxic than LPS, a finding that paved the way towards clinical trials of TLR4 agonists [90].

Clinical activity

Numerous clinical studies examining the adjuvant activity of TLR4 ligands combined with vaccines targeting a wide variety of pathogens and tumor antigens have been conducted (Table 2). In general, adding MPLA to vaccines typically boosted serum Ab titers by 10 - 20 fold when compared to vaccine alone (Table 2). MPLA preferentially induces the production

of IgG2a Abs and supports the generation of Th1 immunity in mice [91;92]. Human vaccine trials indicate that MPLA has a safety profile similar to that of alum [82;92]. Even very high doses of MPLA (up to 100 mg/m^2 delivered i.v.) are safe, inducing neither renal nor hepatic toxicity [93].

Extensive clinical studies were conducted using MPL® (also known as AS04), consisting of MPLA absorbed onto aluminum hydroxide or aluminum phosphate [94;95]. The combination of AS04 plus hepatitis B vaccine (FENDrix®) was studied in immunocompromised individuals (including the elderly, those with immunodeficiency diseases and patients on hemodialysis) [96;97]. FENDrix® was well tolerated and induced higher seroprotection rates and Ab titers than Engerix-B® (the licensed HBV vaccine) in multiple clinical studies [98-102]. In a phase III study [102], the safety and efficacy of 2 doses of FENDrix® was compared to 3 doses of Engerix-B® in >1,300 healthy individuals. One month after the first injection, the seropositivity rate (anti-HBs titer >1 mIU/ml) was 76.8% in the FENDrix® group versus 37.3% in the Engerix-B® group, and seroprotection rates (titer >10 mIU/ml) were 34.1% versus 13.1%, respectively. After the final vaccination, 98.5% of FENDrix® subjects were seroprotected vs 96.8% in the Engerix-B® group. The GMTs elicited by FENDrix® were more than two-fold higher than those elicited by Engerix-B®, a difference that persisted through 36 months [97].

In hemodialysis patients, seroprotection was achieved in 91% of subjects immunized with 4 doses of FENDrix® vs 84% in recipients of Engerix-B® [103]. This differences persisted at 36 months post vaccination (73% versus 52%, respectively). Antibody concentrations in the FENDrix® group were higher at all time points, and the vaccine was well tolerated, resulting in licensure by the EU in 2005.

AS04 was also used as an adjuvant with Cervarix®, a conjugate vaccine that uses virus-like particles to induce immunity against HPV-16 and HPV-18 [94]. When compared to the same vaccine adjuvanted with alum, Cervarix® induced significantly higher anti-HPV16 and anti-HPV18 L1 Ab responses (1.6 - 3.2 fold, $p<0.05$) at all time points [104]. 3.5 years after Cervarix® vaccination, Ab titers were 17 - 30 fold higher than those induced by natural HPV16 or HPV 18 infection [105;106]. The efficacy of Cervarix® was examined in randomized phase II and III clinical studies. In the phase II trial, Cervarix® provided 100% protection against HPV-16/18 infection for >12 months [105]. In the phase III trial (which included women with and without previous HPV exposure), the vaccine reduced the frequency of HPV-associated cervical intraepithelial neoplasia grade 2 by 92.9% [107]. Additional Phase II/III clinical data established the safety and efficacy of Cervarix®, and this AS04-adjuvanted vaccine was approved for use in the EU and the US [107][108;109].

AS04 has also been studied in combination with cancer vaccines. Melacine® consists of the lysates from two allogeneic melanoma cell lines combined with DETOX®, an adjuvant containing AS04 plus cell wall material from *Mycobacterium phlei* [110]. This vaccine was tested in a phase II multi-center clinical trial of melanoma patients with stage III/IV disease [111]. 12% of participants showed some response to immunotherapy, and Melacine® was approved for the treatment of metastatic melanoma by the Canadian FDA [112]. AS04 is also a component of Stimuvax®, a complex mixture of lipopeptides, proteins, and lipids used in patients with adenocarcinomas including non-small cell lung cancer [113;114]. In a phase II trial, Stimuvax® improved median survival time by ≈33% ($p=0.069$) without inducing significant toxicity. A phase III trial to confirm these findings has been initiated [115].

AS04 is heterogenous, in that it contains several different MPLA species that vary in length and degree/type of fatty acid acylation. Building upon the success of MPLA/AS04, a new

class of synthetic lipid A mimetics, the aminoalkyl glucosaminide 4-phosphates (AGPs) was developed [83;92][116]. In contrast to the complex mixture of lipid A congeners found in MPLA, AGPs can be synthesized at high purity as single chemical entities and modified to alter biological/pharmacodynamic activity. RC-529 (also known as Ribi.529) is a leading AGP that is structurally similar to the hexa-acyl component of MPL®. RC-529 was co-administered with the HBV vaccine (Supervax®) and compared to Engerix-B®. Supervax® induced significantly greater seroprotection, achieving 99% seroprotection at 3 months vs 84% for the control group ($p< 0.0001$). Anti-HBV titers were significantly higher at all time points for the Supervax® group. Based on these findings and an acceptable safety profile, the RC-529 conjugated HBV vaccine was approved for use in Argentina [117] .

Safety

In general, adverse events (typically mild - moderate) were more frequent in subjects receiving AS04 or RC-529 adjuvanted vaccines vs alum. In studies of FENDrix®, the frequency of pain, erythema and swelling exceeded that of Engerix-B®, [102]. The safety of the AS04 adjuvanted HPV-16/18 vaccine was summarized in a meta-analysis of 11 clinical trials. Local and systemic adverse events were higher in recipients of the AS04 vs alum adjuvanted vaccine. However compliance with the three-dose schedule did not differ between groups nor did the rates of serious adverse events (2.8% vs 3.1%), medically significant conditions (19.4% vs 21.4%), new onset of chronic diseases (1.7% for both) or new onset of autoimmune diseases (0.4% vs 0.3%) [118]. The safety profile of this TLR4 adjuvant was sufficiently to allow licensure in vaccines used in the US, Europe and Argentina.

In clinical studies of Supervax®, the incidence of injection site pain was significantly increased when compared to the alum-adjuvanted HBV vaccine (70% vs 42%, $p<0.0001$). These local reactions were typically mild to moderate, and resolved within 3 days. The incidence of systemic adverse events was low, and did not differ significantly between groups. No serious systemic adverse events related to vaccination were reported.

TLR5

General overview

TLR5 is triggered by a region of the flagellin molecule expressed by nearly all motile bacteria [119;120]. TLR5 is found on the surface of many types of immune cells, including monocytes, mDCs, Langerhans cells, T cells and NK cells [121-126]. When used as an adjuvant, flagellin is typically fused to a recombinant vaccine Ag. In that form, flagellin directly induces DC maturation, triggering the up-regulation of co-stimulatory signals and Ag-presenting molecules (CD80, CD83, CD86, MHC class II, TNFα, IL-8, IL-1β, CCL2, CCL5) [125]. The effect of fusing flagellin to protein-based vaccines was examined using the fluorescent protein EGFP as a model system. Almost 50% of APCs internalized flagellin-EGFP vs 3% internalizing EGFP alone. Flagellin-EGFP also stimulated APCs to produce 20-fold more TNFα than EGFP ($p<0.001$), and uniquely induced Ag-specific CTL responses *in vivo* [127]. When used as an adjvuant, flagellin stimulates monocytes to produce the cytokines IL-10 and TNFa [123], NK cells to produce IFNg and α-defensins, and T cells to proliferate and produce cytokines and chemokines (e.g. IL-10, IL-8 and IFNg) [122].

TLR5 ligands and their pre-clinical activity

The adjuvant properties of flagellin-Ag complexes were investigated in several animal models. When coupled to ovalbumin, flagellin induced an IgG Ab response 10-fold higher than OVA co-administered with alum [128]. Fusing the poorly immunogenic M2e protein

from the influenza virus with recombinant *Salmonella Typhimurium* flagellin (STF2) yielded a vaccine that induced a 10-fold higher Ag-specific IgG response than M2e formulated in alum [129]. Other studies showed that flagellin-Ag vaccines induced protective Ab responses against *Yersinia pestis* [130], West Nile virus [131], vaccinia virus [132] and *Pseudomonas aeruginosa* [133;134]. These protective responses were characterized by high titered Ag-specific IgG (IgG_1, IgG_{2a}) responses that were generally two logs higher than Ag alone or Ag + flagellin (not fused) ($p<0.05$). Since TLR5 is not expressed on B cells [124]) this enhanced Ab production was presumably mediated by improved APC function.

There is no published information on the immunogenicity or safety of flagellin-based vaccines in humans, although several clinical trials are ongoing. No injection site inflammation or severe systemic adverse effects were detected in studies of mice and non-human primates [130;135].

TLR7/8

General overview

TLRs 7 and 8 are phylogenetically and structurally related [136]. Both recognize single stranded (ss) RNA sequences containing poly-U or GU-rich sequences and are activated by synthetic imidazoquinolines including imiquimod (R-837) and resiquimod (R-848) as well as by guanosine analogues such as loxoribine [136-139]. TLR 7/8 molecules are localized to the endosomal compartments of human immune cells including DCs, monocytes, macrophages, lymphocytes, Langerhans cells, and NK cells [124;140;141]. Because of their numerous similarities, TLRs 7/8 will be handled together in this section.

The interaction between TLRs 7/8 and their cognate ligands activates DC to i) enhance expression of co-stimulatory molecules (e.g. CD80, CD86, CD40) [142], ii) migrate [143] and iii) produce pro-inflammatory cytokines including IFNα, TNFα and/or IL-12 (the later facilitating induction of Th1-type responses). In this context, TLR7-specific activation preferentially triggers IFNα secretion by pDC whereas TLR8-specific activation preferentially induces IL-12 production by mDCs [140;144]. TLR 7/8 also promote the maturation of Langerhans cells and their migration from the skin to the lymph nodes [145;146].

In addition to activating DCs, TLR 7/8 stimulate B cells to secrete Ig and produce cytokines (e.g. IL-6, TNFα) [147] while triggering NK cells to produce IFNg [148]. TLR 7/8 ligation stimulates T cells to proliferate and produce IFNg, IL-2 and IL-10. Memory T cells are particularly sensitive to this form of TLR-mediated activation [122], and TLR7/8 ligands may reduce the immunosuppression mediated by CD4+ T regulatory cells [149].

TLR7/8 ligands and their pre-clinical activity

Most pre-clinical studies involving TLR7/8 agonists utilized imiquimod (which predominantly activates TLR7) or resiquimod (which triggers both TLR7 and 8). Compounds that selectively activate TLR7 (852A) or TLR8 (3M-002) [140;150] have been identified but there is insufficient published information to assess their utility as vaccine adjuvants. It is currently unclear whether ligands targeting TLR7 vs TLR8 will be superior vaccine adjuvants. Resolving this issue through pre-clinical studies is complicated, as there is disagreement in the literature concerning the ability of mice to respond to TLR8 ligands that are active in Man [136;151-153]. Studies utilizing monocytes and APCs collected from human newborns and adults indicate that the Th1-response induced by TLR8 stimulation substantially exceeds that induced by other TLR ligands (including TLR7). This finding

raises the possibility that TLR8 ligands might be of particular utility in vaccines targeting newborns [154;155].

Pre-clinical studies indicate that imidazoquinolines can improve both the magnitude and quality of Ag-specific T cell and Ab responses. In mice and macaques, i.m. and s.c. delivery of TLR7/8 agonists with the HIV-1 Gag protein enhanced Ag-specific IgG and CTL responses, particularly when the adjuvant was conjugated to Ag [156-159]. In a guinea pig model of genital HSV infection, an HSV-2 glycoprotein based vaccine combined with imiquimod enhanced Ab production by up to 10-fold ($p < 0.001$) and reduced the number of lesions and frequency of genital HSV recurrences by >80% ($p<0.001$). Interpretation of these findings was complicated by the observation that imiquimod alone reduced HSV recurrences by 62%, so that no significant reduction for the vaccine + imiquimod vs imiquimod alone groups was observed [160].

TLR7/8 agonists have also been used as adjuvants in combination with cancer vaccines. In a genetically engineered mouse model, a DNA vaccine encoding HER2/neu adjuvanted with imiquimod significantly delayed the development of spontaneous mammary tumors and reduced their incidence by 65% when compared to the DNA vaccine alone. These effects were accompanied by a significant increase in Ag-specific Ab production (3 fold, $p < 0.05$), a switch from IgG1 to IgG2a Ab isotype, and a 30% increase in CTL activity ($p < 0.05$) [161].

Clinical activity

Imiquimod (Aldara, 3M) is the only TLR7/8 agonist to undergo extensive clinical testing (Table 3). It is licensed for topical use to treat warts caused by HPV, basal cell carcinoma, and actinic keratosis [162-165]. Topical imiquimod was tested in combination with several cancer vaccines (Table 3). When used with a vaccine composed of several melanoma peptides plus Flt3 ligand (hematopoietic growth factor), imiquimod increased the fraction of patients who developed peptide-specific CTL responses. However, no effect on disease progression was observed in this study of 27 patients with stage II-IV post surgical disease [166].

Another vaccine used the NY-ESO-1 protein in combination with topically administered imiquimod for the treatment of malignant melanoma (stage II-III). Applying imiquimod cream to the vaccine site stimulated novel Ab and/or $CD4^+$ T cell responses in approximately half of the trial participants. Biopsies of imiquimod treated skin showed a significant enhancement of mononuclear cell infiltrates including T cells, APCs and NK cells vs untreated skin [167].

A phase I-II trial compared topical imiquimod to 3 other adjuvants (GM-CSF, hyperthemia, mucin-1-mRNA/protamine complex) in combination with a multi-peptide vaccine (HLA-A2 restricted TAA-eptiopes and MHC class II-binding peptides) in prostate cancer patients. Recipients of the imiquimod-adjuvanted vaccine showed the best response, as determined by a slowing in the rate of PSA rise. Unfortunately, this study did not evaluate immunological markers or disease progression, so no definitive conclusions can be drawn [168].

In summary, preclinical studies suggest that TLR7/8 ligands can boost both humoral and cell mediated responses to vaccines targeting infectious diseases and cancer. Only imiquimod was clinically evaluated as a vaccine adjuvant, where it boosted Ag-specific Ab and CD4 responses in cancer patients, although it is unclear whether this impacted disease progression.

Safety

Topical administration of imiquimod in human vaccine trials was well tolerated. Adverse events were uniformly mild, transient, and primarily involved local reactions (such as cutaneous erythema). No severe systemic events were reported. These findings are consistent with large clinical trials in which cutaneous imiquimod cream was used to treat diseases such as actinic keratosis [165].

In contrast, severe side effects were reported after oral or systemic use of imidazoquinoline as a monotherapy in humans [169-171]. In a phase I clinical trial of cancer patients, oral administration of 25 - 200 mg of imiquimod was associated with sustained dose-related hematological toxicity. At doses ≥50 mg, grade 3-4 lymphopenia developed in nearly half of all patients. At higher doses, hepatic and renal impairment were found [171]. Thus while safe for topical use, formulating imiquimod with vaccines designed for internal use is likely to be problematic.

TLR 9

General overview

TLR9 is expressed by human B cells and pDC. The receptor is localized within endo-lysosomal compartments and detects the unmethylated CpG motifs present at high frequency in bacterial (but not mammalian) DNA [172]. The recognition of CpG DNA by cells expressing TLR9 has a cascading effect on the immune system, leading to the maturation, differentiation, and/or proliferation of NK cells, T cells, B cells, monocytes and macrophages [124;172-179]. The resultant immune response is characterized primarily by the production of pro-inflammatory and Th-1 biased cytokines (including IL-1, IL-6, TNFα, IFNg and IL-12 [172;173;180-184].

TLR9 ligands

The immunostimulatory activity of bacterial DNA is mimicked by synthetic oligonucleotides (ODN) expressing CpG motifs [185-187]. These are typically composed of phosphorothioate nucleotides, which are considerably more resistant to nuclease digestion than native phosphodiester nucleotides, and thus have a substantially longer half-life *in vivo* [188]. Three major classes of CpG ODN have been described, each with distinct structural and biological properties. “K” type ODN (also referred to as “B” type) consist of multiple CpG motifs on a phosphorothioate backbone. They are strong modulators of B cell activation and induce the maturation of pDCs and monocytes [189-191]. “D” type ODN (also referred as “A” type) are constructed of a mixed phosphodiester/phosphothioate backbone, contain a single CpG motif flanked by palindromic sequences, and a poly G tail at the 3' and 5' termini, facilitating the formation of concatamers. “D” ODN excel are triggering pDC to produce IFNα [182][190]. “C” type ODN resemble “K” type in being composed entirely of phosphorothioate nucleotides, but resemble “D” type in containing palindromic CpG motifs. They have immunostimulatory properties found in both “K” and “D” type ODN, including the ability to activate B cells and stimulate the production of IFNα by pDC [188].

Preclinical studies conducted in rodents and non-human primates demonstrate that CpG ODN can accelerate [192], increase the magnitude [192-194], and prolong the duration of vaccine-specific Ab responses [194-196]. In addition, CpG ODN improve the response induced by mucosal vaccines [197-200] and enhance the immunogenicity of vaccines administered to immunocompromised populations [201-204]. CpG ODN promote vaccine immunogenicity by improving Ag uptake by professional APC (particularly pDC), triggering the functional maturation of APC, and generating a cytokine/chemokine

microenvironment supportive of Ag-specific immunity [205]. These effects are optimized when vaccine and adjuvant are presented to the immune system in close spatial and temporal proximity [193;206;207].

Clinical activity

CpG ODN as adjuvants for vaccines targeting infectious diseases—Human clinical trials examining the adjuvant activity of TLR9 ligands have focused on "K" type ODN. Those trials evaluated CpG ODN combined with vaccines designed to prevent malaria [208-211], hepatitis B (HBV) [212-217] influenza [218] and anthrax [219] (Table 4). When co-administered with Engerix-B®, CpG ODN significantly improved the Ab response of healthy individuals through 48 weeks of follow-up when compared to the conventional vaccine. The geometric mean titer of anti-HBs antibody in volunteers treated with CpG ODN plus Engerix-B® was 13-fold higher after primary and 45-fold higher after secondary immunization than that induced by vaccine alone. Moreover, all participants immunized with the CpG-adjuvanted vaccine developed seroprotective titers by 2 wk after priming [215;216], compared to none of the control group. These findings were replicated in an independent clinical trial [217]. All patients receiving the CpG adjuvanted vaccine maintained titers in the seroprotective range for >1 year compared to 63% vaccinated with Engerix-B® alone [212;213]. The adjuvanted vaccine also induced protective Ab titers in HIV patients who were hyporesponsive to Engerix-B® alone [212].

CpG ODN were also tested as adjuvants in combination with AVA, the licensed anthrax vaccine. The adjuvanted vaccine boosted anthrax specific Ab responses of healthy subjects by 6-8 fold and accelerated the induction of immunity by approximately 3 wk [219]. Other trials showed that naive volunteers mounted a significantly stronger Ab response to the poorly immunogenic malaria vaccine candidates Apical Membrane Antigen 1 (AMA1) and Merozoite surface protein 1_{42} ($MSP1_{42}$) when co-delivered with CpG ODN. The co-administration of CpG with AMA1 increased the GMT of anti-AMA1 Abs by 5.5 fold when compared to subjects receiving AMA1 alone [208]. This enhanced Ab response was achieved using a 4-fold lower concentration of AMA1 and was persistent: at 236 days after vaccination, those immunized with AMA1 + CpG ODN maintained serum Ab titers 4.6-fold higher than those vaccinated with just AMA1 [208]. While challenge studies were not conducted, sera from volunteers vaccinated with AMA1 + CpG ODN was >4-fold more effective at inhibiting the growth of *P. falciparum* 3D7 parasites *in vitro* that those vaccinated with AMA1 alone ($p < .0001$). Unfortunately, the impact of adding CpG ODN was insufficient to overcome the lack of immunogenicity of AMA1 when administered to semi-immune adults with a history of multiple previous plasmodium infections and circulating AMA1-specific Abs [209]. When co-administered with $MSP1_{42}$, CpG ODN boosted average Ab titers by 8 fold when compared to $MSP1_{42}$ alone measured 2 wk after third immunization [211]. The effect of CpG ODN on a vaccine containing both AMA1 and MSP1 is underway (ClinicalTrials.gov Identifier:NCT0088961). The effect of adding CpG ODN to the Fluarix influenza vaccine was less impressive. In that trial, CpG ODN reduced the dose of vaccine required to achieve a strong immune response, but did not increase the magnitude of the response [218].

CpG ODN as adjuvants for vaccines targeting cancer—The goal of most cancer vaccines is to generate large numbers of tumor-specific CTL, as cellular rather than humoral immunity is believed to play a central role in tumor eradication. In pre-clinical animal studies, CpG ODN enhanced the production of cytotoxic $CD8^+$ T cells targeting tumor Ags [220;221]. This effect was observed when ODN were conjugated to or simply co-administered with tumor Ag [222;223]. Of considerable interest, CpG adjuvanted tumor vaccines effectively eliminated established cancers in murine models [224;225].

These findings supported clinical studies using CpG ODN as adjuvants for cancer vaccines (Table 4). In a trial examining the effect of a melanoma Ag A based vaccine (Melan-A; identical to MART-1), inclusion of CpG ODN plus incomplete Freund's adjuvant generated a stronger and more rapid $CD8^+$ T cell response than the unadjuvanted vaccine. In that study, 4 immunizations with the adjuvanted vaccine resulted in >3% of circulating $CD8^+$ T cells being Melan-A-specific: an order of magnitude higher than in patients treated with vaccine alone [226].

In a phase I trial of patients with stage III/IV NY-ESO-1Bexpressing melanoma, only a weak immune response was elicited by vaccination with the NY-ESO-1 peptide plus CpG ODN. When the co-adjuvant Montamide was added to the the NY-ESO peptide plus CpG ODN, the combination promoted the development of Ag-specific $CD8^+$ T cells within 1 month [223]. A separate uncontrolled clinical trial used recombinant NY-ESO-1 protein plus CpG ODN and Montamide to vaccinate patients with different tumor types [227]. Tumor specific Ab responses rose significantly within 6 weeks while cross-primed NY-ESO-1-specific $CD8^+$ T cells were detected in a subset of patients by 12 weeks.

A Phase I study involving 14 patients with different types of cancer detected new Ag-specific $CD8^+$ T cell responses in 9 patients following combined vaccination with NY-ESO-1, CpG ODN and Montanide [228]. Six of these 9 patients lived an average of 39 months, far longer than their predicted survival of only 4 months. Whether vaccine-induced immunity was responsible for this improved clinical outcome could not be determined in this uncontrolled study. In contrast, when CpG ODN was co-administered with GM-CSF and a peptide corresponding to the immunodominant epitope from the tumor antigen hTERT (human telomerase reverse transcriptase), no beneficial effect on the CTL response of patients with sarcoma or glioblastoma was detected [229].

In toto, the clinical data indicates that CpG ODN are likely to find use as vaccine adjuvants, particularly for those vaccines targeting infectious diseases. This reflects the consistent ability of CpG ODN to boost Ag-specific humoral immunity in naive subjects. The utility of CpG ODN as tumor vaccines adjuvants is less clear, as the magnitude of the CTL response needed to clear an established tumor may exceed that which can be induced by the current generation of vaccines. This is particularly true given the ability of tumor cells to suppress or circumvent immune recognition. These concerns have focused attention on the use of CpG ODN in combination with other immunomodulatory agents, an area of research that deserves further attention.

Safety

Toxicity has not been observed in animal studies of CpG adjuvanted vaccines. At much higher doses (used for other purposes), or in combination with agents that induce the production of TNFα (such as LPS or D-galactosamine) [186;230;231], toxicity has been reported. Evidence from clinical trials indicates that CpG ODN are reasonably well tolerated when administered as vaccine adjuvants. However the frequency and severity of local adverse events (injection site reactions such as pain, swelling, induration, pruritus, and erythema) and systemic symptoms (including flu-like symptoms) were elevated. This higher frequency and severity of AEs is likely attributable to the immunostimulatory properties of CpG ODN. Most of these adverse events were mild-to-moderate, appeared within 24 hours of dosing, and persisted for only a few days.

Conclusions

Toll-like receptors differ from one-another in location (intra-cellular vs plasma membrane), use of accessory molecules to induce signaling (TIRAP, TRIF, TRAM and MyD88), the

type of pathogen associated molecules they recognize (nucleic acids, polypeptides, lipopolysaccharides), and the type of response they induce (inflammatory, Th1 or Th2). These distinctions underlie inherent differences in the ability of speicifc TLR ligands to influence the nature of the adaptive immune response they support when used as vaccine adjuvants.

Clinical trials involving TLRs 2, 3, 4, 7/8 and 9 support the broad conclusion that TLR ligands can be safe and effective vaccine adjuvants, with vaccines already licensed in the US, Europe and Argentina containing such ligands. Sadly, there are no head-to-head pre-clinical (much less clinical) trials examining the relative adjuvanticity of different TLR agonists. Since vaccine development is a highly empirical process, and different types of response are required to protect against distinct pathogens/tumors, there is little justification for concluding that one particular ligand will be significantly more useful than other available alternatives. However as clinical data accumulate, evidence of safety and immunogenicity may shift the balance to favor incorporation of one or small subset of TLR agonists in future vaccines.

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Table 1

Overview of published clinical trials utilizing TLR2-dependant adjuvants

Treatment	Disease	Study Phase	Study population	Outcome Reference
OspA + Pam3Cys +/- alum	Lyme disease	Phase III	Healthy Adults	>75% elicit seroprotection additional alum had no benefit [29;30]
OspA + Pam3Cys	Lyme disease	Phase I/II	Children < 15 years	>90% elicit protective IgG response [232;233]
$(T1BT)_4$ + Pam3Cys	Malaria	Phase I	Healthy Adults	All volunteers developed Ag-specific IgGs (2,750GMT), comparable to earlier study using alum /Q21 [33]
Lipo-6 +/- QS-21	HIV	Phase I/II	Healthy Adults	HIV-specific B and T cell responses in up to 93% and 86% of volunteers, respectively, QS-21 had no benefit [38;39;234235]
Lipo-4	HIV	Phase I	Healthy Adults	I.d. injection required only 20% of the i.m. dose and induced CTL response in up to 52% volunteers [236]
Lipo-6	HIV	Phase II	HIV-infected adults	New $CD4^+$ and $CD8^+$ T cell responses in 70% and 57% of patients, respectively, leading to longer HAART interruption [237;238]
Lipo-6T+ IL-2/vCP1433	HIV	Phase II	HIV-infected adults	No impact on HIV-specific $CD4^+$ T cells and only transient effect on CTL [40]
Theradigm-HBV	HBV	Phase-I	Healthy Adults	Dose dependant HBV-specific CTL response persisting for > 9 months [35;36]
Theradigm-HBV	HBV	Phase II	HBV-infected adults	No effective CTL response [37]
HPV-16 E7	HPV	Phase I	Cervical/vaginal cancer patients	No effective CTL response [239]

OspA; outer-surface protein A, Pam3Cys; tri-palmitoyl-S-glyceryl cysteine, $(T1BT)_4$; repeat T and B cell epitopes + universal T cell epitope of the *P. falciparum* CS protein, Lipo-4; mixture of 4 Nef/Pol/ Nef lipopeptides, Lipo-6; mixture of 6 Nef/Gag/ Env lipopeptides, Lipo-6T; mixture of 6 Nef/Gag/Pol lipopeptides, vCP1433; recombinant canarypox vector, Theradigm-HBV; contains a HTL epitope (tetanus toxin peptide) covalently linked to a CTL epitope (HBV core peptide) palmitoylated at the N terminus, HPV-16 E7; lipidated HPV-16 E7 peptide epitope linked to PADRE (HTL epitope)

Table 2

Overview of published clinical trials utilizing TLR4-dependant adjuvants

A. Infectious Diseases					
Treatment	**Disease**	**Study Phase**	**Study population**	**Outcome Reference**	
AS04 + HbsAg	Hepatitis B	Phase III	>1,300 healthy adults	Nearly 100% seroprotection and 2-fold higher Ab titers than control vaccine	[102]
AS04 + HbsAg	Hepatitis B	Phase II	165 hemodialysis patients	Seroprotection elicited more rapidly and effectively	[103;240]
AS02 (MPL + QS21) + rHBV	Hepatitis B	Phase I/II	30 healthy adults	Induced strong Ab and CTL response	[241]
AS02 (MPL + QS21) + HbsAg	Hepatitis B	Phase I	20 liver transplant recipients	80% of patients had a strong Ab response allowing them to suspend HBIG therapy	[242]
RC-529 + alum + HbsAg	Hepatitis B	Phase III	285 healthy adults	Seroprotection at day 90 was 99% for RC-529 and 84% for controls ($p<0.0001$)	[117]
AS04 + HPV-16/18 VLPs	Genital HPV	Phase III	>18,500 healthy women	93% prophylactic efficacy against HPV-induced intraepithelial neoplasias	
AS04 + HPV-16/18 VLPs	Genital HPV	Phase III	>650 healthy women	100% seropositivity after 3 doses	[108]
AS04 + HPV-16/18 VLPs	Genital HPV	Phase II	>1,100 healthy women	100% protection for 1 yr, remained seropositive for >7 yr	[105;106;243;244]
AS04 + HPV-16/18 VLPs	Genital HPV	Phase II	129 healthy women	Ab titer higher ($p<0.05$) for up to 2 yr	[104]
AS04 + HSV glycoprotein D	Genital HSV	Phase III	>2,700 healthy adults	74% protection for HSV-1/2 seronegative subjects	[245]
AS04 + gp350	EBV	Phase II	181 healthy adults	99% with anti-gp350 Abs for >18 mos	[246]
AS04 + LEISH-F1	Leishmaniasis	Phase I/II	80 healthy adults	IFN-γ response and DTH to LEISH-F1	[247]
AS02 (MPL + QS21) + NefTat / $gp120_{W61D}$	HIV	Phase I	84 healthy adults	HIV-specific lymphoproliferation	[248]
AS02 (MPL + QS21) + RTS,S	Malaria	Phase II	306 healthy men	71% efficacy against infection for 9 wk	[249]
AS02 (MPL + QS21) + RTS,S	Malaria	Phase II	>2,000 healthy children	Infection reduced 29% for 21 mos	[250;251]
AS02 (MPL + QS21) + Mtb72f	Tuberculosis	Phase I	12 healthy adults	Increased IL-2 and IFN-γ production	[252]
B. Cancer					
Treatment	Disease	Study Phase	Study population	Outcome Reference	
Detox adjuvant + lysates from two melanoma cell lines	Melanoma	Phase II	139 melanoma patients	Clinical response rates were CR 3%, PR 5%, MR 4%, and PD 65%	[111]
Detox adjuvant + lysates from two melanoma cell lines	Melanoma	Phase III	140 melanoma patients	No difference in response rates or survival	[253]
Detox adjuvant + lysates from two melanoma cell lines	Melanoma	Phase III	553 melanoma patients	5-year relapse-free survival rate was 83% in patients who matched more than 2 of the M5[1]	[254]
Detox adjuvant + Stn-KLH	Breast cancer	Phase II	23 breast cancer patients	All patients developed Abs to STn	[255]

A. Infectious Diseases

Treatment	Disease	Study Phase	Study population	Outcome	Reference
MPL + BLP25 + three lipids[2]	Lung cancer	Phase II	171 NSCLC patients	Median survival time extended to 17.4 vs 13.0 months (p=0.065)	[256]
MPL + BLP25 + three lipids[2]	Prostate cancer	Phase II	16 prostate cancer patients	50% had stable PSA	[257]
MPL + QS21) + MAGE-3 protein	MAGE-3 positive metastatic cancer	Phase I/II	57 cancer patients	96% developed anti-MAGE-3 Abs	[258;259]
AS02 (MPL + QS21) + MAGE-3 protein	MAGE-3 positive lung cancer	Phase II	17 NSCLC patients	Elicited strong MAGE-3 specific Ab and $CD4^+$ T-cell response	[260]

MPL; monophosphoryl lipid A, alum; aluminum hydroxide or aluminum phosphate, LEISH -F1; recombinant *Leishmania* polyprotein (formerly known as Leish-111f), NefTat; comprised of Nef (derived from HIV_{LAI}) and Tat (derived from HIV_{BH10}), RTS,S; fusion protein of the carboxyl-terminal half of the *P. falciparum* (strain NF54, laboratory clone 3D7) circumsporozoite protein, Mtb72f; recombinant protein comprising two antigens (Mtb39a and Mtb32a) expressed in *M. tuberculosis* and in BCG,

Table 3

Overview of published clinical trials utilizing TLR7/8-dependant adjuvants

Treatment	Disease	Study Phase	Study population	Outcome Reference	
K562 cells transfected with GM-CSF +/- imiquimod	CML	Phase II	Patients with CML	Reduction of tumor burden and improved molecular response. Additional imiquimod had no benefit.	[261]
HPV16 E6E7L2 fusion protein + imiquimod	Vulval cancer	Phase II	Patients with VIN (grade II/III) HPV +/-	63% were lesion responders with local $CD4^+$ and $CD8^+$ T cell infiltration. No control group without imiquimod included.	[262]
Prostate specific peptides + montanide +/- imiquimod	Prostate cancer	Phase I/II	Patients with PC (stage II/III)	Imiquimod-adjuvanted vaccine slowed the PSA rise better than other adjuvants.	[168]
NY-ESO-1 + imiquimod	Melanoma	Phase I	Patients with stage IIB-III melanoma	50% developed Ag-specific Ab and/or $CD4^+$ responses. No $CD8^+$ responses.	[167]
melanoma peptides (Tyr, Mel, Ny-ESO-1) +Flt3/ +/- imiquimod	Melanoma	Phase I	Patients with stage II-IV melanoma	62.5% of patients developed Ag-specific CD8+ T cells when imiquimod was applied, in contrast to 25% without imiquimod.	[166]

[1] M5; any of 5 HLA class I Ags (HLA-A2, HLA-A28, HLA-B44, HLA-B45, and HLA-C3), [2] the three lipids are cholesterol, dimyristoyl phosphatidylglycerol and dipalmitoyl phosphatidylcholine, Detox adjuvant; includes monophosphoryl lipid A and cell wall skeleton from *Mycobacterium phlei*, STn-KLH; STn is a cancer-associated core region carbohydrate antigen of epithelial mucin and STn linked to keyhole limpet haemocyanin (KLH), BLP25; 25-amino acid sequence that provides MUC1 antigen, NSCLC; non small cell lung cancer, BSC; best supportive care, PSA; prostate specific antigen, MAGE-3; melanoma antigen-3, PC; prostate carcinoma, VIN; vulval intraepithelial neoplasia, PSA ; prostate specific antigen, NY-ESO-1; cancer-testis Ag, Flt3; hematopoietic growth factor.

Table 4

Overview of published clinical trials utilizing TLR9-dependant adjuvants

A. Infectious Diseases					
Treatment	**Disease**	**Study Phase**	**Study population**	**Outcome Reference**	
CpG 7909 + AMA1/AlhydrogelH	Malaria	Phase I	Healthy Adults	5.5 fold increase in anti-AMA1 Ab titer	[208]
CpG 7909 + AMA1/AlhydrogelH	Malaria	Phase I	Semi-Immune Adults	2 fold increase in Ab response,	[209;210]
CpG 7909 + $MSP1_{42}$	Malaria	Phase I	Healthy Adults	Ab titers increased 8-fold after 3 doses	[211]
CpG 7909 + Engerix-B®	Hepatitis B	Phase I/II	HIV-infected Adults	100% serocoversion after 6 wk (vs 63% in controls), seroprotection maintained 5 yr	[212;213]
CpG 1018 + HBsAg	Hepatitis B	Phase III	Healthy young adults	Seroprotection achieved faster and and with fewer doses than Engerix-B®.	[217]
CpG 7909 +	Hepatitis B	Phase I/II	Healthy adults	Seroprotection achieved faster, and anti-HBs Ab titers 45-fold higher post boost vs Engerix-B®.	[214;215] [216]
CpG 7909 + Fluarix	Influenza	Phase I	Healthy adults	Immune response not enhanced, but reduced vaccine doses without reduced immunogenicity required	[218]
CpG 7909 + AVA	Anthrax	Phase I	Healthy adults	Ab response increased 7-fold, and accelerated by 3 wk.	[219]
B. Cancer					
Treatment	Disease	Study Phase	# of subjects/group	Outcome Reference	
CpG 7909 + Melan-A/MART-1	Melanoma	Phase I	8 patients with melanoma	10-fold increase in Melan-A specific $CD8^+$ T cells (>3%)	[226]
CpG 7909 +NY-ESO-1 protein +/- Montanide	Melanoma	Phase I	8 patients with melanoma	Increased $CD8^+$ T cell response	[223]
CpG 7909 +NY-ESO-1 protein + Montanide	Melanoma Breast cancer Sarcoma Ovarian cancer	Phase I	18 patients with melanoma 14 cancer patients by 6 wk,	Increased Ab and $CD4^+$ Th cell response $CD8^+$ T cell response by 12 wk	[227]
CpG 7909 +NY-ESO-1 protein + Montanide	Melanoma Lung cancer Ovarian cancer Breast cancer Sarcoma	Phase I	13 patients with melanoma cancer patients	Increased $CD8^+$ T cell response and prolonged survival	[228]
CpG 1018 + GM-CSF + hTERT peptide	Sarcoma Glioblastoma	Phase I	17 cancer patients	$CD8^+$ T cell response in only 1 subject	[229]

AMA1; apical membrane antigen 1, $MSP1_{42}$; Merozoite surface protein 1_{42}, MART; Melanoma Antigen Recognized by T-cells, hTERT; human telomerase reverse transcriptase