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REVIEW

# Psychopharmacology: neuroimmune signaling in psychiatric disease-developing vaccines against abused drugs using toll-like receptor agonists

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## Abstract

**Rationale** Since substance use disorders have few or no effective pharmacotherapies, researchers have developed vaccines as immune-therapies against nicotine, cocaine, methamphetamine, and opioids including fentanyl.

**Objectives** We focus on enhancing antibody (AB) production through stimulation of toll-like receptor-5 (TLR5) during active vaccination. The stimulating adjuvant is Entolimod, a novel protein derivative of flagellin. We review the molecular and cellular mechanisms underlying Entolimod's actions on TLR5.

**Results** Entolimod shows excellent efficacy for increasing AB levels to levels well beyond those produced by anti-addiction vaccines alone in animal models and humans. These ABs also significantly block the behavioral effects of the targeted drug of abuse. The TLR5 stimulation involves a wide range of immune cell types such as dendritic, antigen presenting, T and B cells. Entolimod binding to TLR5 initiates an intracellular signaling cascade that stimulates cytokine production of tumor necrosis factor and two interleukins (IL-6 and IL-12). While cytokine release can be catastrophic in cytokine storm, Entolimod produces a modulated release with few side effects even at doses 30 times greater than doses needed in these vaccine studies. Entolimod has markedly increased AB responses to all of our anti-addiction vaccines in rodent models, and in normal humans.

**Conclusions** Entolimod and TLR5 stimulation has broad application to vaccines and potentially to other psychiatric disorders like depression, which has critical inflammatory contributions that Entolimod could reduce.

**Keywords** Entolimod · Toll-like receptor-5 · Substance use disorder · Vaccine

## Anti-drug vaccines: immunotherapy for substance use disorders

Increasing research on treating substance use disorders (SUDs) has considered not only neurobiological, but also immunological approaches for these chronic medical conditions. Moreover, approved and effective pharmacotherapies continue to be limited to only a few specific substances such as opioids and alcohol. Therefore, investigators have examined immunological approaches for SUDs including active immunization with vaccines (Martell et al. 2009; Esterlis et al. 2013; Haile et al. 2015), passive immunization with anti-drug monoclonal antibodies (mAB) (Stevens et al. 2014; Harris et al. 2015; Kvello et al. 2016), administration of metabolic enzymes to inactivate abused drugs (Nasser et al. 2014; Cohen-Barak et al. 2015), and gene transfer of antidrug proteins including mAB and metabolic enzymes (Hicks et al. 2012; Rosenberg et al. 2012; Murthy et al. 2016; Smethells et al. 2016).

The theoretical basis for the anti-addiction vaccines is that the production of adequate levels of specific antibodies (AB) can capture and sequester the drug of abuse in the peripheral circulation, as the large AB-drug compound molecules cannot cross the blood-brain barrier, thereby diminishing the physical and psychological reinforcing effects of the drugs. Even if the AB can only hamper the speed of the drug entry without fully preventing them from entering the brain, the reinforcing effects of the drugs are reduced (Woolverton and Wang 2004; Nelson et al. 2006; Schindler et al. 2009). The diminished reinforcing effects

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subsequently lead to reduced drug use. These vaccines also can block the priming effect of drug use and prevent the conversion of drug use lapses into full relapses. These vaccines can usually produce sufficient AB levels to block modest doses of the abused substances (Martell et al. 2009; Haney et al. 2010). However, they are unlikely to block purposeful high-dose use, in which the abuser wants to over-ride these AB, unless the drug is highly potent and very small amounts produce substantial effects. For example, the fentanyl class of opioids is highly potent and typical AB levels elicited from fentanyl vaccine could probably block even overdoses with relatively large fentanyl doses (Bremer et al. 2016; Raleigh et al. 2019).

As the drug molecules are not inherently immunogenic, vaccines for SUDs consist of drug-based haptens conjugated to immunogenic carrier proteins (e.g., keyhole limpet hemocyanin, cholera toxin, or tetanus toxoid) combined with adjuvants to increase immunogenicity. Several studies have demonstrated reduced brain/plasma ratios of the drug in vaccinated or passively immunized animals compared to controls (Hieda et al. 1997; Keyler et al. 1999; Pravetoni et al. 2012a, b). Subsequently, studies of vaccines for nicotine, cocaine, heroin, and methamphetamine (MA) showed promising anti-addiction effects. Preclinical studies of these vaccines were reviewed in detail elsewhere (Ohia-Nwoko et al. 2016; Pravetoni 2016). To date, vaccines against nicotine and cocaine have undergone human clinical trials. While several of these first-generation formulations demonstrated efficacy in the subset of patients with the highest anti-drug AB concentrations, post-immunization serum AB response varied substantially among individuals, with only about half of the subjects attaining clinically effective AB responses (Martell et al. 2009; Hatsukami et al. 2011). Given the high degree of variability in individual responses, overall results have been modest, and no SUD vaccines to date have demonstrated efficacy in phase III trials.

Despite the lack of demonstrated efficacy from the pooled data, cocaine and nicotine anti-addiction vaccines can be efficacious when the vaccine produces sufficiently high AB levels (Cornuz et al. 2008; Martell et al. 2009; Hatsukami et al. 2011). But the required AB concentrations for effective vaccination against SUDs are much higher than that needed for adequate immunization against microbe/toxin in an infectious disease. Peak plasma cocaine concentrations are ~ 500 nM in a typical recreational cocaine abuser who takes a dose of 40 mg free-base cocaine (Jenkins et al. 2002). A vaccine that elicits anti-cocaine AB exceeding 250 nM (~ 40 μg/mL), equal to 500 nM antibody binding sites, would be able to sequester a large fraction of that dose. However, in published clinical trials of both cocaine (Martell et al. 2009) and nicotine (Hatsukami et al. 2011) conjugate vaccines, one half to two thirds of patients receiving vaccine did not produce the adequate concentration of AB to effectively reduce CNS drug uptake. Additionally, the AB concentration rapidly diminishes within a few months after the initial booster doses in the clinical trials for cocaine and nicotine vaccination (Martell et al. 2009; Hatsukami et al. 2011). Therefore, the blocking of drugs in even the high AB level responders was too brief to be optimally beneficial, and patients would need repeated boosters every 2 to 3 months.

## Approaches to improve antibody quantities

Researchers have developed various strategies to increase AB production by SUD vaccinations including improvements in immune-adjuvants, carriers, hapten, and hapten and carrier protein linker design, as well as the incorporation of multivalent immunization strategies and novel particle-based delivery systems, reviewed elsewhere (Ohia-Nwoko et al. 2016; Pravetoni 2016). For carriers, the responses in mice to keyhole limpet hemocyanin succinylnorcocaine (KLH-SNC) and tetanus toxoid succinylnorcocaine (TT-SNC) using an alum adjuvant are substantially higher than that to cholera toxin B (CTB)-SNC in both initial and later response periods (Orson et al. 2014). Three MA vaccines using KLH as a carrier protein have generated substantial AB concentrations with good affinity for MA (Moreno et al. 2011). The TT protein carrier conjugated to succinyl-methamphetamine (SMA) has produced stronger immune responses than a vaccine with a diphtheria toxoid carrier (Collins et al. 2016). Current strategies to modify the hapten and linkers are aiming to enhance the affinity and specificity of the anti-drug AB (Cai et al. 2013a, b; Ramakrishnan et al. 2014).

The simplest approach to improve immunity might be applying adjuvants, which are primarily derived from microbial components and include killed mycobacteria, such as complete Freund's adjuvant, Bordetella pertussis toxin, extracts of Toxoplasma gondii, Mycobacterium-derived muramyl peptides, lipopolysaccharide (LPS) or its toxic components, lipid A and CpG–rich DNA motifs (Akira et al. 2001). The immune system recognizes the structurally conserved molecules on the bacteria, virus, and other microbes through the pattern recognition receptors (PRRs). The toll-like receptors (TLRs) constitute a subgroup of PRRs that recognize the microbial antigens (Mogensen 2009). Macrophages, endothelial and epithelial cells, and dendritic cells (DCs) all express TLRs. These receptors rapidly respond to the pathogens through the activation of an array of immune cells and through AB production (Honko and Mizel 2005). Thus, AB production can be improved by targeting TLRs via adjuvants, which potently engage both the innate and adaptive immune responses (Loré

et al. 2003; Giannini et al. 2006; Cooper et al. 2008; Didierlaurent et al. 2009; Søgaard et al. 2010).

## Targeting the TLR system to improve antibody production of anti-drug vaccines

In the presence of TLR agonists, antigen presenting cells (APC) undergo a process of maturation, which renders an initially non-immunogenic antigen immunogenic and allows the immune system to effectively respond to pathogenic antigens (Blander and Medzhitov 2006; Garaude et al. 2012). The enhanced immunity by TLR agonists is highly relevant to anti-drug vaccine immunity. With the presence of TLR agonists, the immune cells are sensitized to the encounter with the novel non-endogenous stimulus, which is the abused drug attached to a portion of an inherently immunogenic protein such as tetanus toxoid. In this way, adjuvants based on the TLR system can increase the potency of an anti-drug vaccine through pre-activation of the immune system to respond with the cascade of processes leading to a specific AB response.

Different TLR agonists have been tried in various vaccines: TLR-9 (CpG ODN 1826), TLR-4, and TLR-2 (Duthie et al. 2011; Bremer et al. 2014; Collins et al. 2016). The TLR-9 adjuvant is DNA based. The TLR-2 and TLR-4 adjuvants are based on various lipids: lipopolysaccharide, liposomes containing monophosphoryl lipid, lipid A derived from Gram-negative bacteria, and glucopyranoside lipid A (Ishizaka and Hawkins 2007; Morefield et al. 2007; Lockner et al. 2013; Stevens et al. 2016). One of the most effective vaccines, the live attenuated yellow fever vaccine 17D (YF-17D) is able to activate multiple TLRs (2, 7, 8, and 9) on DCs to elicit a broad spectrum of innate and adaptive immune responses (Querec et al. 2006).

The TLR-5 adjuvant, Entolimod, is a pharmacologically optimized protein derivative from flagellin protein in *Salmonella thyphimurium*, retaining the two constant regions of flagellin essential for TLR5 binding (Burdelya et al. 2008). Studies have established Entolimod as a potential treatment for lethal radiation exposure, for which it has an excellent safety profile (Burdelya et al. 2008; Vijay-Kumar et al. 2008). Entolimod can alleviate bone marrow and intestinal injuries in mice and rhesus monkeys by stimulating stem cells and thereby promoting regeneration of radiosensitive tissues (Jones et al. 2011; Krivokrysenko et al. 2015). This review examines the possible cellular role of the adjuvant Entolimod (also known as CBLB502), in enhancing the efficacy of anti-drug vaccines and discusses its application in anti-drug vaccine development.

TLR systems have gained attention as promising therapeutic targets to stimulate antitumor immunity by initiating innate responses and subsequent adaptive T cell-based immunity, as reviewed previously (Akira et al. 2001; Kanzler et al. 2007). However, the activation of many TLR systems could lead to a cascade of systemic cytokine release (cytokine storm), which can be life-threatening (Islam et al. 2016; Voss et al. 2016; Murakami et al. 2017; Perrin-Cocon et al. 2017). In addition, even relatively lower levels of cytokine activation often produce a variety of psychiatric symptoms, which has limited the application of cytokine and TLR activation treatments in psychiatric patients (Dantzer and Kelley 2007). The TLR5 system activation with Entolimod seems to have unique advantages over the activation of other TLR systems in psychiatric patients, because of Entolimod's safety profile even with systemic delivery. Interestingly, there are no endogenous ligands that bind to TLR5, and flagellin is its only known natural ligand (Hayashi et al. 2001). Flagellin, when used as a fusion protein with particular antigens or as a separate adjuvant combined with vaccines, has shown great potency in generating antibodies in both animal studies and clinical trials (Taylor et al. 2011; Holbrook et al. 2016; Mardanova et al. 2016; Labastida-conde et al. 2018). For example, for an anti-cocaine vaccine in mice, using flagellin protein itself as the carrier rather than an adjuvant, these cocaine-flagellin conjugates enhanced the dose-dependent production of anti-cocaine AB better than other protein carriers (Lockner et al. 2015).

Flagellin binding to TLR5 initiates an NF-κB-mediated signal transduction cascade and stimulates productions of TNF-α, IL-6, and IL-12 (Eaves-Pyles et al. 2001). This response is thought to contribute to the promotion of both innate and humoral responses (McSorley et al. 2002; Honko et al. 2006a; Sfondrini et al. 2006). The maximum tolerated dose of flagellin is relatively limited, as AB response towards flagellin itself markedly reduces its ability to enhance AB responses to other antigens. In contrast, Entolimod can be as efficacious as flagellin to induce NF-κB nuclear translocation, yet elicit a significantly weaker AB response to itself (Burdelya et al. 2008). More importantly, in all tested species, including humans, Entolimod led to robust production of a spectrum of cytokines with particular hematopoietic and immunity regulatory implications, but minimal or absent induction of cytokines implicated in cytokine storm (Gribble et al. 2007; Tarrant 2010).

In addition to its role in enhancing AB production against foreign antigens, Entolimod has protective effects that likely vary in different diseases. These protective mechanisms can involve TLR5-dependent NF-kB-mediated induction of anti-apoptotic pathways, of scavengers for reactive oxygen species, of cytokines, and of anti-inflammatory agents (Burdelya et al. 2008, 2013; Fukuzawa et al. 2011). Practical applications of these multiple mechanisms have been tested in tumor immunity. Entolimod exerts antitumor effects in several tumor models (Sfondrini et al. 2006; Rhee et al. 2008; Cai et al. 2011; Burdelya et al. 2012) and has significantly improved the survival of mice with metastatic tumors upon the treatment with donor-derived immune cells (Ding et al. 2012).

Overall, activation of TLR5 by flagellin or Entolimod triggers a robust immunological response that engages a broad range of immune cell types that participate in both innate and adaptive immune systems (Honko and Mizel 2005; Mizel and Bates 2010; Hossain et al. 2014; Brackett et al. 2016; Shim et al. 2016).

## Cellular mechanisms of enhanced immunity by Entolimod

### Enhanced innate immunity by Entolimod mediated through the activation of natural killer (NK) cells

Entolimod can engage both natural killer (NK) cell-dependent innate immunity and T cell-dependent adaptive immunity pathways. The NK cells play an important role in innate immune responses that are non-specific to any particular pathogen and may contribute to anti-pathogen immunity as well as to the side effects of local inflammation at the site of vaccinations. This rapid, non-specific innate immunity effect of Entolimod has important therapeutic and preventative applications. For example, single doses of Entolimod administered 48 to 72 h prior to murine cytomegalovirus (CMV) infection protected mice against CMV lethality. This protection from CMV was unrelated to AB production and adaptive immunity, but instead was due to Entolimod enhancing innate immunity by stimulation of TLR5 receptors on NK cells and thereby enhancing the cytotoxic activity of these cells (Hossain et al. 2014). In addition, flagellin promotes several innate immune processes, including the induction of pro-inflammatory cytokines and chemokines (Means et al. 2003; Rolli et al. 2010; Kozlova et al. 2014; Ma et al. 2016) and recruitment of immune cells to peripheral lymphoid sites (Bates et al. 2008; Flores-langarica et al. 2012). These innate immune processes involve a variety of flagellin-responsive cell types (e.g., DCs, epithelial cells, and lymph node stromal cells) (Sanders et al. 2008). Entolimod interacts directly with TLR5 on these immunological cells leading to their maturation. In two syngeneic lymphoma models, Leigh et al. showed that Entolimod directly activated TLR5-expressing CD11b+ and CD11c+ accessory cells, provoking an immunological microenvironment conducive to enhanced immunogenicity against any antigen entering that microenvironment. This microenvironment, in turn, stimulates a robust immune response (and potentially vaccine AB) by NK cell-dependent immunity and activation of CD8+ cytotoxic T cells (Leigh et al. 2014). The above innate immune processes enhanced by flagellin or Entolimod are the crucial priming for the AB production during the adaptive immune response that follows vaccination.

### Entolimod enhances adaptive immunity

Entolimod's enhancement of AB production from vaccines largely involves adaptive immunity, which is a slower but more sustained process leading to specific immunological memory. Induction of the innate immunity via flagellin/Entolimod, as discussed above, contributes to maximum antibody productions. It is likely that only a threshold level of innate immunity triggered by flagellin/Entolimod can drive the antigen-specific humoral response (Honko et al. 2006b; Bates et al. 2008).

While flagellin or Entolimod-induced DC activation is the indirect mechanism enhancing AB production (Salazar-Gonzalez et al. 2007; Ding et al. 2012), recognition of flagellin by TLR5 on the DCs can also directly trigger the antigen-specific differentiation of naive B cells into plasma cells and differentiation of T helper cells, both of which are important components of antibody-producing adaptive immunity (Uematsu et al. 2008). Numerous studies have demonstrated that flagellin significantly enhances T cell-dependent AB production (Honko et al. 2006b; Holbrook et al. 2016; Qian et al. 2016; Kim et al. 2018). In addition, Entolimod engages chemotactic actions in these adaptive immune responses. For example, Entolimod drives the recruitment of NK cells to the liver of animals with liver metastatic tumors (Brackett et al. 2016) and the activation of DCs by NK cells. Flagellin can also markedly induce the recruitment of T and B lymphocytes to draining lymph nodes (Bates et al. 2008), which increases the likelihood of antigen-specific lymphocytes encountering their cognate antigen. These chemotactic effects of Entolimod are relevant to anti-addiction vaccination as Entolimod attracting immune cells to the site of vaccination will enhance the vaccine's AB production.

The establishment of an antigen-specific durable immune memory for AB production is the basis for the antitumor (Burdelya et al. 2013; Yang et al. 2015) and antiviral efficacy (Hossain et al. 2014) of vaccines adjuvanted with Entolimod. This enhancement of AB responses also occurs in humans (our unpublished data). Based on its efficacy to enhance antigen-specific immune memory in animal tumor models and humans, we postulated that Entolimod could also enhance vaccine-specific immune memory and enhance AB production in an anti-addiction vaccine. Entolimod significantly increases AB levels beyond those produced using conventional adjuvants such as aluminum (Kim et al. 2018). The synergism of aluminum and Entolimod probably resulted from aluminum inducing a Th2 (T helper cell type 2) response of the "depot" effect and from Entolimod triggering TLR-5 activation of the MyD88 (myeloid differentiation primary response 88) and TRIF (TIR-domain-containing adaptor-inducing interferon-β) dependent pathways leading to Th1 (T helper cell type 1) responses through IL-12p70 induction (Netea et al. 2005; Stills 2005; Khong and Overwijk 2016). This mixed Th1/Th2 response was observed in the staphylococcal

enterotoxin B vaccine co-administered with aluminum and a TLR adjuvant (Morefield et al. 2007). Both Th1 and Th2 cells support antigen-specific B cell expansion and AB production, contributing to the enhanced AB production (Smith et al. 2000). These polyclonal AB also showed a broad but acceptable range of AB affinities (20 nM to 110 nM) (Miller et al. 2013). In rodent studies, anti-MA monoclonal ABs with Kd affinity values for MA of 20 nM can be sufficient for MA binding that substantially reduces MA brain entry and MA-induced behaviors (Carroll et al. 2009).

In berief, Entolimod adjuvant activates immune cells in complex interacting cascades to enhance AB production, subsequently increasing the overall potency of Entolimod/flagellin-based vaccines. These interacting cascades await further exploration.

## Entolimod suppresses immune-mediated diseases

Flagellin and its derivative Entolimod are potent activators of innate and adaptive immunity and enhance AB and antitumor immunology, as reviewed above. Paradoxically, in inflammatory disease, Entolimod decreases immune reactions, therefore mitigating inflammatory diseases such as hepatitis. Entolimod pretreatment completely protected mice from anti-Fas AB-induced fulminant liver injury by increasing hepatocyte resistance (Burdelya et al. 2013). Entolimod's protective role in attenuating hepatitis in these Entolimod pretreated mice depended on the MyD88/NF-κB signaling pathway. Using the concanavalin A (Con A)-induced hepatitis mouse model, Wang et al. found that Entolimod acted as a negative regulator limiting T cell/NK T cell activity and pro-inflammatory cytokine production (Wang et al. 2017). Suppressive activities of flagellin on NK T cells were also demonstrated in both animal (Kim et al. 2008) and human studies (Shim et al. 2016). In the in vitro experimental study, flagellin treatment of the peripheral blood mononuclear cell in asthma patients suppressed the circulating Th2- and Th17-like invariant natural killer T (iNKT) cells in an IL-10-dependent mechanism, indicating the immunomodulatory role of flagellin (Shim et al. 2016).

This anti-inflammatory role of Entolimod as a negative regulator of cytokines may be more broadly relevant to psychiatric disorders, to which cytokine activation contributes (Dantzer and Kelley 2007). Entolimod's AB enhancing properties can increase the potency of monoclonal AB or vaccine-induced AB against specific pro-inflammatory cytokines such as tumor necrosis factor (TNF). The pathophysiology of major depressive disorders involves inflammation related to TNF, and monoclonal ABs against TNF in particular have successfully reduced symptoms in patients with treatment-resistant depression (Raison et al. 2013).

## Potential enhancement of Tregs by Entolimod

Regulatory T cells (Tregs), formerly known as suppressor T cells, are immune-suppressive and generally suppress or down-regulate the induction and proliferation of effector T cells. Tregs express high levels of TLR5 mRNA, and treatment of Tregs with flagellin enhances the regulatory activity of these cells (Crellin et al. 2005). In the experimental models of Con A-induced hepatitis, Entolimod pretreatment increased the numbers of Tregs of the CD4 + CD25 + FoxP3+ types (Wang et al. 2017). These Tregs protect Con A-induced hepatitis by suppressing immune responses. The relevance of this Treg suppression to optimizing AB production is that transient suppression of Tregs at an appropriate time point can boost the maximal AB level, as has been studied in tumor vaccination (Elia et al. 2007; Rech and Vonderheide 2009; Rolla et al. 2010). Manipulation of Tregs can improve the immunization potential of various anti-tumor vaccines. If Entolimod enhances the activity of Tregs at appropriate time points, this might be yet another pathway that enhances AB responses through TLR5 on Tregs.

# Future directions

Several vaccines have used Entolimod as a separate adjuvant and shown excellent efficacy in animal models. Using Entolimod itself as the protein carrier might further improve this efficacy. Flagellin fusion proteins have elicited markedly enhanced AB responses, roughly 10-fold greater than those vaccines using flagellin as a separate adjuvant. This enhancement was likely due to the more efficient delivery of adjuvant and antigen to the same TLR5+ APC (Mizel and Bates 2010). In another study, flagellin-functionalized nanoparticles are noted to have more pronounced immunostimulation than free flagellin (Kozlova et al. 2014). Thus, potential future strategies to further enhance the adjuvant activity of Entolimod in anti-addiction vaccines may include the development of covalent conjugates of drug haptens with Entolimod as the carrier protein or calcium phosphate nanoparticles loaded with flagellin/Entolimod, which may further increase the AB quantity.

As discussed in this article, Entolimod, as well as its precursor flagellin, can both promote immunity and dampen immunity in different contexts. The molecular and cellular mechanisms for TLR5 stimulation on a wide range of immune cell types are complex, and they remain only partially understood. In the literature, both tumor-promoting and antitumor immunity, as well as pro-inflammatory and anti-inflammatory effects, have been linked to TLR5 (Honko and Mizel 2005; Shim et al. 2016). Further exploration of Entolimod's role in suppressing and stimulating networks of the immune system clearly will advance vaccine research in addictions and develop new approaches to immunotherapies for a broadening range of psychiatric disorders.

## Compliance with ethical standards

**Conflict of interest** On behalf of both authors, the corresponding author states that there is no conflict of interest. Our vaccine studies are in collaboration with Cleveland Biologic Labs (CBLI) who are providing Entolimod free of charge.

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