Oral subunit vaccine design

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Many pathogens invade the host at the intestinal surface. To protect against these enteropatho-gens, the induction of intestinal secretory IgA (SIgA) responses is paramount. While systemic vaccination provides strong systemic immune responses, oral vaccination is the most efficient way to trigger protective SIgA responses. However, the development of oral vaccines, especially oral subunit vaccines, is challenging due to mechanisms inherent to the gut. Oral vaccines need to survive the harsh environment in the gastrointestinal tract, characterized by low pH and intestinal proteases and need to reach the gut-associated lymphoid tissues, which are protected by chemical and physical barriers that prevent efficient uptake. Furthermore, they need to surmount default tolerogenic responses present in the gut, resulting in suppression of immunity or tolerance. Several strategies have been developed to tackle these hurdles, such as delivery systems that protect vaccine antigens from degradation, strong mucosal adjuvants that induce robust immune responses and targeting approaches that aim to selectively deliver vaccine antigens towards specific immune cell populations.

oral vaccination subunit vaccines mucosal immunity

1. Introduction

Vaccines play a crucial role in reducing the global burden of infectious diseases and are responsible for the elimination of diseases like polio, tetanus and pertussis and even the eradication of smallpox and rinderpest [1][2][3][4]. Furthermore, vaccines can aid in solving the current crisis regarding antimicrobial resistance by eliminating or reducing the need for antibiotics, especially in animal husbandry [5][6]. Most vaccines are administered via parenteral routes, generally leading to strong systemic immune responses. In contrast, most pathogens infect or invade the host at mucosal surfaces and systemic immunity generally does not provide sufficient protection against these types of pathogens. Local administration of vaccines to mucosal surfaces, such as via oral vaccination, provides much better protection against pathogens that colonize or invade these surfaces by inducing mucosal immunity, characterized by the local production of secretory IgA (SIgA) as well as a systemic immunity [7]. The production of SIgA is crucial because it shows improved stability in the gut via its secretory component and can prevent the colonization of the gut tissues by pathogens, such as enterotoxigenic *Escherichia coli* (ETEC), via a process called “immune exclusion”, characterized by agglutination, entrapment and clearance of the pathogen [8]. In addition to eliciting robust intestinal immune responses, oral vaccination has other advantages over parenteral vaccines, such as the reduced need for trained personnel, allowing self-administration, and a reduced risk of transmitting blood-borne diseases due to needle-free administration. They also increase patient compliance due to easier administration and often do not require refrigerated storage, which results in easier transport and delivery to remote places. For most oral vaccines, no expensive purification techniques or equipment are required, generally

making it easier to get market approval. Finally, they also have a more cost-effective production, drastically reducing the cost of mass vaccination programs [9][10]. Currently, most oral vaccines consist of either inactivated or live-attenuated organisms. The latter have several risks attributed to them, such as uncontrolled replication, severe inflammatory reactions, the risk of infection in immunocompromised patients and the possibility of reversion to a virulent strain. In recent years, the focus for oral vaccination strategies has shifted to the use of safer subunit vaccines, but these still face many hurdles. Oral vaccine antigens have to survive the harsh environment of the gastrointestinal tract, characterized by a low gastric pH and degradation by gastric and small intestinal proteases. They also have to be able to reach the gut associated lymphoid tissue (GALT), which is protected by an epithelial barrier that has evolved to regulate nutrient absorption as well as to provide protection against foreign invaders [9]. Furthermore, under normal circumstances, antigens that enter via the oral route are treated as dietary components. If a vaccine does not induce the appropriate danger signals, then the gut tissues will recognize it as non-pathogenic, resulting in suppression of immunity or tolerance [11][12]. Compared to parenteral immunizations, high dosages are generally required for successful immunization, but these larger doses also increase the risk of tolerance [13][14][15]. Because of this risk, inclusion of potent adjuvants is essential for promoting robust intestinal immune responses [16]. The limited residence time of vaccine antigens in the gut is also an important factor to consider as it can prevent their effective uptake [17]. All these obstacles generally lead to poor immune responses to oral vaccination and are the main reasons so few effective oral vaccines exist. Finally, the microbiota might also impinge on the efficacy of oral vaccines. Currently, this aspect of oral vaccination is not yet well understood it certainly requires further research [18][19].

2. Oral Vaccination Strategies

Several oral vaccination strategies have been developed in recent years to tackle the different hurdles associated with oral vaccination. Potent oral adjuvants have been developed that can stimulate the mucosal immune system and are capable of provoking robust mucosal immune responses. Different delivery systems have been designed that are able to protect vaccine antigens against the harsh gastrointestinal environment and release these antigens at the immune inductive sites to promote uptake by antigen presenting cells. Furthermore, by targeting specific receptors, selective delivery of vaccine antigens towards specific cell populations within the intestinal tissues can be achieved, further promoting robust intestinal immune responses. These oral vaccination strategies will be addressed in the next sections and are briefly summarized in Table 1.

Table 1. Overview of different oral vaccination strategies studied for oral administration.

Oral adjuvants	
Toxin derivates	dmLT [20][21][22][23], mmCT [24]
PRR ligands	β-glucans [25][26], MPL [27], Flagellin [28][29], CpG [30][31]

NKT-ligands	α-galactosyl ceramide [32][33]
Delivery systems	
Living delivery systems	Recombinant bacteria [34][35][36][37][38][39][40][41][42][43][44] Viral vectors [45][46][47][48][49][50][51][52][53][54][55][56]
Non-living delivery systems	Virus-like particles [57]
	Micro- and nanoparticles [58][59][60]
	Lipid-based delivery systems [61][62][63][64][65][66][67][68][69]
	Nanogels [70]
Targeted delivery	
M-cells	Dectin-1 [71], GP2 [72], C5aR [73]
Enterocytes	FcRn [74] Aminopeptidase N [75][76][77][78][79]

2.1. Oral Adjuvants

As mentioned, most licensed oral vaccines make use of complete live-attenuated or inactivated organisms, which do not require potent adjuvants. These types of vaccines present an inherent adjuvanticity through the presence of conserved molecular patterns, such lipopolysaccharide (LPS), flagellin or cytosine-phosphate-guanine (CpG), which are recognized by pathogen recognition receptors (PRR)[80]. Since oral subunit vaccines generally do not possess any adjuvant functions, the addition of potent mucosal adjuvants is required to circumvent the default tolerogenic responses present in the gut and to allow the induction of robust intestinal immune responses [81][82].

Several types of adjuvants can be distinguished, and these can be broadly divided into two groups: the immunopotentiators and the delivery systems. Generally, immunopotentiators have the ability to enhance the immune response against otherwise weak immunogenic antigens and result in a broad and durable protection, while delivery systems improve the vaccine delivery to the targeted site or help protect the antigen from degradation. Often a combination of both these systems is used by including immunopotentiators in the delivery system or because the delivery system itself has inherent immune stimulating properties [83][84][85].

2.1.1. Toxin Derivates

One of the most important classes of immunopotentiators for oral vaccination is toxin derivates, such as the ADP-ribosyl transferase enterotoxins cholera toxin (CT) and heat-labile enterotoxin (LT). Due to their strong adjuvant properties and their ability to elicit SIgA, they are considered the gold standard for oral vaccination [86][87]. These toxins stimulate antigen-presenting cells, enhancing the expression of MHC class II and costimulatory molecules, and induce antigen-specific T_H2 and T_H17 cells to secrete IgA-promoting cytokines, further supporting the production of IgA [88][89]. It has been shown that oral delivery of CT to mice activates the canonical NF-κB pathway and mRNA expression of NF-κB-dependent pro-inflammatory cytokines in the mesenteric lymph nodes and Peyer's patches [90]. Their effects are also thought to promote the permeation of antigens across the epithelial barrier and to promote intestinal stem cells to differentiate into M cells, an epithelial cell specialized in the uptake of macromolecules [91][92][93].

Although LT and CT are often used as potent oral adjuvants in animal models during preclinical research, unfortunately they display a high toxicity in humans, resulting in severe diarrhea at low doses and thus preventing their use as oral adjuvants in humans. Fortunately, modified versions of these toxins, such as the double mutant LT (dmLT) and multiple-mutated CT (mmCT), have been developed in recent years, resulting in a decreased toxicity, while retaining their potent adjuvant properties. In mice, dmLT has been shown to be an effective mucosal adjuvant when given orally together with several antigens from different pathogens, often providing protection against subsequent challenge infection. In humans, a live-attenuated ETEC vaccine (ACE527) was co-administered with dmLT, resulting in protection after subsequent ETEC challenge. The oral inactivated ETEC vaccine ETVAX also showed increased immune responses in children and infants, but not in adults when adjuvanted with dmLT.

An alternative strategy to co-administration would be to conjugate or fuse these toxins to non-immunogenic antigens, allowing the binding of the B-subunit to intestinal epithelial cells, resulting in uptake and transport through the epithelium and improved immunogenicity. Examples of this strategy in the literature include fusion proteins, such as fimbriae-toxin multi-epitope fusion antigens (MEFA) [94][95][96][97][98][99]. Although these showed promising results in inducing protective immunity after i.m and s.c. administration in mice, the protective efficacy of this vaccination strategy still needs to be assessed after oral administration and challenge infection.

Besides LT and CT, the potential use of other toxin derivates, such as adenylate cyclase toxins, as an adjuvant for oral vaccination still has to be further investigated. In mice, nasal co-delivery of the anthrax edema toxin with

ovalbumin resulted in high antigen-specific IgG and IgA serum responses and induced antigen-specific T-cell secretion of IFNγ, IL-5, IL-6 and IL-13 [100].

2.1.2. PRR Ligands

Pathogen recognition receptors play a crucial role in the recognition of pathogens and the induction of appropriate immune responses. They are expressed by many cell types, including intestinal epithelial cells and antigen presenting cells, such as dendritic cells and macrophages. In mice, the expression of some Toll-like receptors (TLR) by intestinal epithelial cells seemed to be age-dependent and differed along the length of the intestine, with the expression of TLR5 being restricted to Paneth cells in the small intestine and gradually decreased during the neonatal period [101]. PRR ligands have been intensively investigated for their adjuvanticity and can be subdivided in the membrane-bound TLRs and C-type lectin (CLR) receptors and the cytoplasmic RIG-I-like (RLR) and NOD-like (NLR) receptors (Table 2).

Table 2. Overview of pathogen recognition receptors, with their respective cellular localization and ligands.

Pathogen recognition receptor (PRR)	Cellular localization	Ligand
Toll-like receptors (TLR) [102][103][104][105][106]		
TLR1	Plasma membrane	Peptidoglycans/lipoproteins
TLR2	Plasma membrane	Peptidoglycans/lipoproteins
TLR3	Endosome	dsRNA
TLR4	Plasma membrane	LPS
TLR5	Plasma membrane	Flagellin
TLR6	Plasma membrane	Lipoproteins
TLR7	Endosome	ssRNA
TLR8	Endosome	ssRNA

TLR9	Endosome	Unmethylated CpG
TLR10	Endosome	Unknown
NOD-like receptors (NLR) *[107][108]		
NOD1/2	Cytoplasm	Peptidoglycans
NLRP3	Cytoplasm	PAMP, DAMP **
NLRC4	Cytoplasm	Cytosolic flagellin
C-type lectin receptors (CLR)[109]		
Dectin-1	Plasma membrane	β-glucans
Clec9A	Plasma membrane	F-actin
DC-SIGN	Plasma membrane	Mannose
Mannose receptor	Plasma membrane	Glycans
RIG-I-like receptors (RLR)[110]		
RIG-I	Cytoplasm	dsRNA
MDA-5	Cytoplasm	dsRNA

* Many more NLRs exist (NLRP1-14, NLRC1-5, NAIP, CIITA), but most of these have not been extensively researched [111]. ** Many different pathogen and damage associated molecular patterns are able to activate the NLRP3 inflammasome. This has been excellently reviewed by Kelley et al. [112].

The ligands of CLR, NLR and RLR have not been studied well as oral adjuvants. So far, only β-glucans have been shown to have immune stimulating properties after oral administration. The potential adjuvanticity of several TLR-ligands, such as monophosphoryl lipid A (MPL; TLR4), flagellin (TLR5) and CpG (TLR9) has been better studied [113][114]. MPL is a detoxified derivative of LPS [114]. Its interactions with TLR4 triggers the production of TNFα, IL-12 and IFNγ, promoting T_H1 immune responses. In mice, pulmonary immunity against *M. tuberculosis* was obtained after oral administration of *M. tuberculosis*-derived antigens with MPL-based adjuvants . The TLR5 ligand flagellin is a highly abundant protein in flagellated bacteria and promotes the induction of pro-inflammatory cytokines and chemokines, the recruitment of B- and T-cells to secondary lymphoid tissues, the direct activation of T-cells and the activation of DC's [115][116]. Flagellin produced in plants was shown to be a potent adjuvant after oral administration with ovalbumin in mice. Flagellin-coated ovalbumin-containing nanoparticles were found to enhance SIgA antibody responses to ovalbumin after oral administration in mice. In humans, an influenza-flagellin fusion vaccine (VAX125) provided strong systemic immune responses after intramuscular immunization [117][118]. CpG is a synthetic oligodeoxynucleotide composed of unmethylated CG motifs. Its binding to TLR9 triggers the secretion of pro-inflammatory and T_H1-specific cytokines by DC's, facilitating the induction of cell-mediated immunity. CpG also promotes the maturation and proliferation of NK cells, T-cells and monocytes/macrophages [119][120]. In mice, oral administration of purified hepatitis B surface antigen or tetanus toxin adjuvanted with CpG provided both systemic and mucosal immune responses. In piglets, oral vaccination with a live-attenuated pseudorabies virus, adjuvanted with CpG, resulted in significantly higher serum IgG and mucosal IgA responses compared to piglets that did not receive the adjuvant. Antigen-presenting cells have also been found to make a distinction between living or dead cells via TLR8-dependent detection of bacterial RNA, resulting in the differentiation of follicular T-helper cells. TLR8-agonists, such as CL075 or R848 showed similar responses and might hold promise as oral adjuvants [121].

An important observation that argues against the use of PRR ligands for oral vaccination is that the intestine already continuously encounters these ligands, which could result in hypo-responsiveness or the presence of a higher threshold for PRR ligand-mediated cellular activation.

2.1.3. Other Immune Modulating Molecules

Other immune modulating molecules that have been investigated as adjuvants include Natural Killer T (NKT) ligands and stimulator of interferon genes (STING) ligands. NKT ligands, such as the synthetic α-galactosyl ceramide, activate NKT-cells by binding to the CD1d receptor on antigen presenting cells. This α-galactosyl ceramide-CD1d complex is subsequently recognized by the NKT T-cell receptor. Mucosal tissues contain many NKT-cells that secrete both T_H1, T_H2 and T_H17-specific cytokines upon stimulation. Alpha-galactosyl ceramide has been shown to be an effective adjuvant, inducing mucosal and systemic cell-mediated immunity after nasal or oral delivery with HIV peptide antigens in mice [122]. Addition of α-galactosyl ceramide to the oral cholera vaccine Dukoral® also strongly enhanced intestinal immune responses in mice.

STING ligands, such as cyclic dinucleotides of bacterial origin (2',3'-cGAMP, 3',3'-cGAMP, c-di-AMP and c-di-GMP), can stimulate robust type 1 interferon responses and proinflammatory cytokines, such as TNFα, IL-1β and IL-6, resulting in the activation of macrophages and dendritic cells. These primarily showed promising results with intranasal use and it would be interesting to assess their efficacy in oral vaccination [123][124].

2.1.4. Use of Adjuvants for the Induction of SIgA after Parenteral Administration

Two factors are crucial for inducing the production of SIgA at the induction sites. First, cytokines play an important role in driving the differentiation of T-helper cell populations, permitting intestinal immunity. Secondly, gut homing of effector cells, like plasma cells, towards the mucosal effector sites is another crucial step.

Gut homing is orchestrated by the expression of mucosal addressins, integrins, chemokine receptors and their ligands [125][126]. They allow the migration of activated lymphocytes and antibody-secreting cells towards specific regions in the gut. Both the integrin α4β7 and the chemokine receptor CCR9 are known to regulate gut homing of immune cells towards mucosal tissues in the gut [127][128]. The integrin α4β7 is present on activated T and B cells and allows binding to the mucosal vascular addressin cell adhesion molecule-1 (MAdCAM-1), expressed on endothelial cells in the high endothelial venules (HEV) of the small intestine and Peyer's patches [129]. The chemokine receptor CCR9 is also present on T-cells and binds specifically to the chemokine CCL25, expressed within the crypts and lower villi of the small intestinal epithelium and on the surface of vascular endothelial cells in the small intestine [130][131][132]

Successful oral immunization should result in the activation of intestinal dendritic cells that produce high amounts of ATRA, leading to the generation of IgA-secreting cells capable of migrating towards the intestinal mucosa. Several factors influence the immune stimulating effects of ATRA, including IL-5, IL-6 and IL-21 or sphingosine 1-phosphate. Both IL-5 and IL-6 synergistically modulate the IgA producing effects of ATRA by modulating IgA class switching in a T-cell independent manner [135][136][137]. IL-21 production can be triggered by IL-6 and drives plasma cell differentiation. Expression of sphingosine 1-phosphate is regulated by ATRA signaling and is needed for the egression of immune cells from the lymphoid organs into the lymphatic vessels [138][139][140][141][142][143]. Besides gut homing and antibody-class switching, ATRA also promotes the generation of regulatory T cells and inhibits the differentiation of T_H17 cells by enhancing TGFβ signaling [144][145].

Although ATRA is not necessarily considered as a mucosal adjuvant, its function could be important for the development of vaccines aiming at eliciting robust mucosal immune responses. Upon subcutaneous or intraperitoneal administration of ATRA together with vaccine antigens, increased α4β7 and CCR9 expression on lymphocytes and increased T-cell trafficking towards the gut were observed in mice and pigs [146][147][148]. More recently, a vaccine using two liposomal delivery systems that were subcutaneously administered to mice induced antigen-specific intestinal IgA responses. The first delivery system was designed to ensure fast drainage of ATRA towards the lymph nodes to precondition these for mucosal immune responses, while the second delivery system was optimized for slower, prolonged delivery of the antigen to these ATRA preconditioned lymph nodes via

migrating antigen-presenting cells [149]. In the future, it would be interesting to see if similar results could be obtained in large animal models.

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