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MINI-REVIEW

Developing inexpensive malaria vaccines from plants and algae

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Abstract Malaria is a parasitic, mosquito-borne, infectious disease that threatens nearly half of the global population. The last decade has seen a dramatic drop in the number of malaria-related deaths because of vector control methods and anti-malarial drugs. Unfortunately, this strategy is not sustainable because of the emergence of insecticide-resistant mosquitoes and drug-resistant *Plasmodium* parasites. Eradication of malaria will ultimately require low-cost easily administered vaccines that work in concert with current control methods. Low cost and ease of administration will be essential components of any vaccine, because malaria endemic regions are poor and often lack an adequate healthcare infrastructure. Recently, several groups have begun addressing these issues using inexpensive photosynthetic organisms for producing vaccine antigens and exploring oral delivery strategies. Immune responses from plant-based injectable malaria vaccines are promising, but attempts to adapt these for oral delivery suggest we are far from a feasible strategy. Here, we review examples of these technologies and discuss the progress and potential of this research, as well as the obstacles ahead.

Keywords Malaria · Oral vaccine · Algae · Tobacco · Mucosal adjuvant

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Introduction

Human malaria is caused by any of four different species of protozoan parasites from the genus *Plasmodium*: *falciparum*, *vivax*, *malariae*, and *ovale*. *Plasmodium falciparum* is responsible for the majority of the over half a million malaria deaths per year, which are predominantly children under the age of five that live in indigent African nations (WHO 2012). These nations lack sufficient resources to effectively combat malaria, which contributes to continued poverty (Ingstad et al. 2012). Bed nets, insecticides against the *Anopheles* mosquito vector, and anti-malarial drugs have been instrumental in reducing disease burdens over the last decade, but these measures are not sustainable. The incidence of insecticide-resistant mosquitoes (malERA 2011b), drug-resistant malaria (Eastman and Fidock 2009), and counterfeit drugs is increasing (Nayyar et al. 2012). The best way forward is to develop efficacious vaccines, but progress has been slow, primarily because the malaria parasite life cycle is complex (Riley and Stewart 2013), involving several distinct cell types, and evades the immune system through antigenic variation (Kirkman and Deitsch 2012). Complicating matters further, malaria vaccines must be extremely inexpensive to produce and easy to administer. It is not feasible to implement a large-scale vaccination campaign if the cost is similar to recent subunit vaccines like the HPV vaccine ($130/dose (CDC 2013)). Realistically, a malaria vaccine needs to be a tiny fraction of that cost. Reaching these goals requires new strategies for both producing and delivering vaccines.

Individuals that live in regions with high malaria transmission gradually acquire immunity only after repeated infections over an extended period of time (Mueller et al. 2013). These infections induce a diverse set of malaria parasite specific antibodies as well as CD4 and CD8 T cells that together confer

immunity. Paramount to developing malaria vaccines that can elicit a similar immune response is a better understanding of the parasite life cycle (Fig. 1). There are three distinct stages—liver, blood, and mosquito. All three stages are potential points of vaccine intervention (Riley and Stewart 2013). Pre-erythrocytic vaccines target sporozoites, the infectious cells of malaria that are injected into the bloodstream during a mosquito bloodmeal. Early attempts at pre-erythrocytic vaccines demonstrated that radiation-attenuated sporozoites can confer sterile immunity to malaria when injected via mosquitoes (Duffy et al. 2012). A recent study demonstrated that intravenously injected, attenuated, and cryopreserved *P. falciparum* sporozoites can provide complete protection (Seder et al. 2013). While extremely promising, production and administration of sufficient in vitro-cultured sporozoites using this strategy are technically challenging and not scalable using current technology.

Recent work has focused on identifying specific parasite antigens that elicit the desired cellular and humoral immunity (Patarroyo et al. 2012). These subunit vaccines are generally made in recombinant systems, purified, and delivered via injection. Antigens can also be delivered using viral vectors

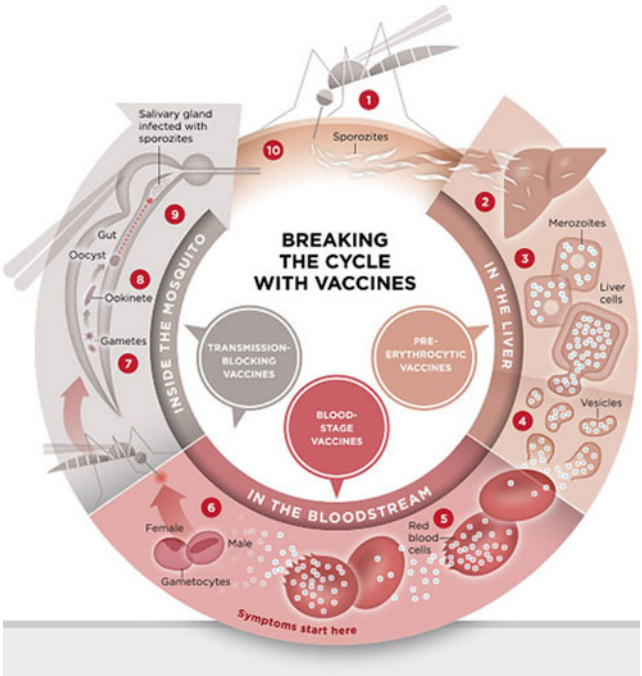

Fig. 1 Malaria parasite life cycle and potential points of vaccine intervention. *1* Mosquito bloodmeal introduced sporozoites into the bloodstream. *2* Sporozoites enter the liver. *3* Asexual division into merozoites within liver cells. *4* Merozoite filled vesicles release parasites into the circulatory system via the lungs. *5* Cycles of red blood cell invasion by merozoites causing repeated bouts of symptoms. *6* A subset of merozoites develop into sexual stage parasites. *7* Mosquitoes take up sexual stage parasites during a bloodmeal. *8* Motile ookinetes burrow through the midgut and develop into oocysts. *9* Thousands of sporozoites travel to the mosquito salivary glands when the oocysts burst. *10* The parasite life cycle repeats after being transferred to a new human host via the mosquito (Image credit: PATH Malaria Vaccine Initiative)

(Hill et al. 2010; Schuldt and Amalfitano 2012). Subunit vaccines are safer than attenuated pathogens because they cannot cause disease and do not require large-scale cultivation of pathogens. The most studied pre-erythrocytic subunit vaccine, RTS/S, uses a 189 amino acid peptide from the circumsporozoite protein (CSP) (Duffy et al. 2012). The aim of this vaccine is to elicit circulating antibodies and a T cell response that confers protection to the vaccinated individual. Thus far, data from phase 3 RTS/S clinical trials suggests modest, but significant protection (RTS/S Clinical Trials Partnership 2012).

Unlike pre-erythrocytic vaccines, blood and mosquito stage vaccines would not prevent initial infection. Instead they would reduce malaria symptoms and transmission, respectively. Sporozoites asexually multiply into merozoites after reaching liver over a 7–10-day period. These cells are released within vesicles that travel through the circulatory system to the lungs where they dissolve and release the merozoites into the bloodstream and attack red blood cells (Baer et al. 2007). Blood stage vaccines focus on merozoite antigens and aim to reduce red blood cell invasion and eliminate intraerythrocytic parasites. Mosquito stage vaccines, more commonly called transmission blocking vaccines (TBVs), focus on antigens from sexual stage parasites (malERA 2011a). Antibodies to several of these proteins block parasite sexual development when taken up with *Plasmodium* gametocytes during a mosquito bloodmeal, thus preventing mosquito infection and subsequent transmission to the next human host. Antibodies raised in mice to TBV candidate antigens have successfully blocked transmission in both animal models and standard membrane feeding assays (SMFA, discussed below), but unfortunately have not advanced beyond safety tests in human clinical trials.

Malaria vaccines from photosynthetic organisms

Production and purification of subunit vaccines are often complex and expensive. Once a promising vaccine antigen has been identified, it is made using one of several possible heterologous systems. Bacteria, yeast, insect, and mammalian cells are most commonly used for producing recombinant proteins, and each has advantages and disadvantages with respect to cost and ease of use. Importantly, the expression platform that is used must be capable of producing a recombinant antigen that faithfully mimics native protein structure. Doing so ensures that the immune response confers protection to the corresponding pathogen. This is difficult to achieve because predicting whether a heterologous platform can replicate the three-dimensional structure of a foreign protein is nearly impossible, particularly for unique or structurally complex antigens, and several malaria vaccine antigens are prime examples of this dilemma. *Plasmodium* proteins are rarely glycosylated and often contain highly repetitive sequences or

complex tertiary structures. For example, the leading TBV candidate, Pfs25, contains tandem repeats of epidermal growth factor (EGF)-like motifs, each containing multiple disulfide bonds, and Pfs25 is not glycosylated. Pfs25 made in *Escherichia coli* did not elicit transmission-blocking (TB) antibodies (Kaslow et al. 1992), most likely because *E. coli* cannot efficiently form disulfide bonds in Pfs25 even when targeted to the periplasm. There are two distinct isoforms of yeast-produced Pfs25, only one of which has the correct conformation, and the primary sequence must be mutated in order to prevent glycosylation (Zou et al. 2003). Merozoite surface protein 1 (MSP1), a blood stage vaccine candidate, also contains EGF-like domains that are required for eliciting protective antibodies (Egan et al. 1996). Correct disulfide bond formation in another blood stage vaccine antigen, apical membrane antigen 1 (AMA1), is required for eliciting protective antibodies (Anders et al. 1998). Unfortunately, alternative mammalian and insect cell platforms are too costly to be considered for producing malaria vaccines.

Photosynthetic organisms, including terrestrial plants and algae, offer several advantages over conventional heterologous protein production systems and are a potential solution (Daniell et al. 2009; Melnik and Stoger 2013). From a production standpoint, they are safe, inexpensive, and easily scalable. Biomass can be generated for pennies per gram or even fractions of a penny in the case of algae (Georgianna and Mayfield 2012), and plant or algae biomass can be freeze dried for long-term storage at ambient temperatures. The absence of human pathogens, prions, or other harmful toxins in many photosynthetic organisms greatly reduces the risk of residual contamination following purification. The earliest examples of plant-produced recombinant proteins were made in tobacco in the late 1980s; plant-produced Hepatitis B virus vaccine followed shortly thereafter (Mason et al. 1992). In 2006, Dow AgroSciences licensed the first plant-produced vaccine. Several more from other groups are presently in clinical trials (Rosales-Mendoza et al. 2012; Yusibov et al. 2011).

After over two decades of research, the close relative of tobacco, *Nicotiana benthamiana*, has established methods for both nuclear and plastid transformation, defined promoters and regulatory elements, and has the highest yields of any plant system to date. Arguably, the most popular transformation method is *Agrobacterium* infiltration of leaf tissue, which uses transient nuclear expression from viral vectors (Giritch et al. 2006). Rather than taking more than a year to generate a stable transgenic plant, recombinant protein can be recovered in less than 1 month. Using viral vectors can cause genetic drift of the transgene over generations, which makes scaling up a significant challenge (Kearney et al. 1993), but academic and commercial entities have made significant progress in recent years (Davies 2010).

Several malaria vaccines have been produced using transient expression or stably transformed plants, including *P. falciparum* blood stage and TBV candidates (Clemente and Corigliano 2012). In studies conducted by Fraunhofer USA, *N. benthamiana*-produced $Pfs25_{22\text{-}193}$ (NbPfs25) (Farrance et al. 2011a) and $Pfs230_{444\text{-}730}$ (NbPfs230) (Farrance et al. 2011b) elicited TB antibodies in mice and rabbits, respectively, as measured by SMFA. More recently, they demonstrated that fusing Pfs25 to virus-like particles can reduce the amount of antigen needed to elicit TB antibodies (Jones et al. 2013). Because *P. falciparum* does not infect mice or other animal models, SMFAs are used to emulate the process of a mosquito bloodmeal. *P. falciparum* gametocytes and sera from vaccinated or unvaccinated animals are added to human blood, which is then fed to female *Anopeheles stephensi* mosquitoes. Mosquito midguts are later dissected and analyzed for the presence of ookinetes; a reduction of oocysts in mosquitoes that received sera from vaccinated animals indicates the presence of TB antibodies.

We recently produced $Pfs25_{22\text{-}193}$ and $Pfs48/45_{178\text{-}448}$ in the chloroplast of the green alga *Chlamydomonas reinhardtii* and, in collaboration with Joseph Vinetz's laboratory at the University of California, San Diego, demonstrated that alga-produced Pfs25 (CrPfs25) elicits TB antibodies by SMFA (Gregory et al. 2012). *C. reinhardtii* is a single-celled eukaryotic alga that has long been used as a model system to study photosynthesis and flagella function, but has only recently been exploited as a platform for producing recombinant proteins. Although still in its infancy compared to *N. benthamiana*, algae have been used to produce industrial enzymes (Rasala et al. 2012), vaccine antigens (Surzycki et al. 2009), and complex immunotoxins (Tran et al. 2013) on an academic scale. Depending on the desired posttranslational modifications, transgenes can be expressed from the nuclear or chloroplast genome. Unlike tobacco and other terrestrial plants, stable transgenic algal strains can be constructed in a few weeks time. They are also easily scaled in fully contained photobioreactors or in outdoor ponds, although large-scale production is currently limited to biofuel and nutraceutical production (e.g., Solazyme, Sapphire Energy, and Nutrex) rather than recombinant proteins.

Expression in *C. reinhardtii* chloroplasts is markedly different than transient nuclear expression in *N. benthamiana.* Chloroplasts have prokaryotic-like ribosomes and importantly, lack the machinery for post-translational glycosylation, thus the peptide sequence of CrPfs25 is identical to native Pfs25. In contrast, NbPfs25 elicits transmission-blocking antibodies only when produced as a fusion with lichenase or when potential N-linked glycosylation sites are mutated (Farrance et al. 2011a). This drawback could potentially be overcome by co-expressing bacterial PNGase F (Peptide: N-glycosidase F) to remove posttranslational glycosylation (Mamedov et al. 2012), but the impact of PNGase on the host cell viability, Pfs25 folding, and the ability of Pfs25 to elicit TB antibodies is unknown. Algal chloroplasts contain

eukaryotic chaperones (Liu et al. 2007), peptidylprolyl isomerases (Ingelsson et al. 2009), and disulfide isomerases (Kim and Mayfield 1997), which presumably facilitate production of the complex EGF-like domains in CrPfs25. A high priority for TBV development should be a head-to-head comparison of TB activity in sera from animals vaccinated with CrPfs25 or NbPfs25 of equivalent purity.

Oral vaccines

Biomass from plants and algae is a promising low-cost feedstock for injectable vaccines, but a large portion of the cost is due to purification, cold storage, and administration by injection. For example, RTS/S is produced in yeast, which is a relatively inexpensive platform, but purification and the complex adjuvant significantly increases production costs (Geels et al. 2011). The ideal malaria vaccine must be extremely inexpensive, heat-stable, and easily administered without a skilled medical worker. Using a whole cell, oral vaccine for malaria could avoid all of these costs. To that end, several groups have begun investigating strategies for plant-based orally available vaccines that require little or no preprocessing, but there are currently no approved oral subunit vaccines, and their development faces immense challenges (Renukuntla et al. 2013). Currently, oral vaccines are available for polio, rotavirus, cholera, and typhoid, but these vaccines are based on attenuated or heat-killed pathogens. Novel strategies are necessary to overcome the obstacles that block orally available subunit vaccines, especially for pathogens like malaria than cannot easily be cultured and primarily affect poor regions of the world.

Antigen uptake by the gut associate lymphoid tissue (GALT) is inefficient partly because of the proteolytic and acidic stomach environment. To ensure bioavailability of orally delivered proteins, antigens must be sufficiently protected by encapsulation, which could be accomplished with either synthetic nanoparticles or biological cells with protective cell walls. Several groups are working to overcome the challenges associated with oral delivery using nanoparticles (Ensign et al. 2012). As this technology develops, it could be a viable option for oral delivery, but antigen–nanoparticle formulation will always be more complex and costly than using whole cells because the payload protein must first be purified from the expression platform. Ideally, proteins for vaccines will be both produced and delivered using the same cells, completely eliminating the cost of purification and formulation.

Many plants and algae could be used as a delivery vehicle for oral vaccines because they are edible, and therefore generally recognized as safe (GRAS) by the US Food and Drug Administration (FDA). GRAS-approved organisms lack microbial toxins and they are not susceptible to human pathogens. The first plant-based oral vaccine, which used tobacco and potato, found that oral administration of plant tissue containing recombinant heat-labile enterotoxin subunit β (LTB) from *E. coli* induces low levels of both serum IgG and secretory IgA (sIgA) antibodies (Haq et al. 1995). Unfortunately, the complexity of the mucosal immune system, which must discriminate between pathogenic and harmless organisms, has slowed oral vaccine development (Mowat 2003). Repeated, large oral doses of antigen prior to systemic immunization can mute T cell-mediated responses to the same antigen. Indeed, the default immune response to most soluble antigens in the gut is one of non-responsiveness or tolerance (Mason et al. 2008). As a result, orally delivered protein subunit antigens elicit little or no adaptive immune response.

Initial strategies to overcome these obstacles combined high doses of plant tissue with co-administered or conjugated mucosal adjuvants, or prior parenteral vaccination. Several of these studies took advantage of the β subunit of the cholera toxin (CTB), a well-characterized mucosal adjuvant, for malaria vaccines (Clemente and Corigliano 2012; Kumar et al. 2012; Kwon et al. 2013). In one example, AMA1 and MSP1 fusions to CTB produced in tobacco chloroplasts elicited antibodies in mice that inhibited parasite invasion of human red blood cells in vitro and provided protection against cholera toxin challenge (Davoodi-Semiromi et al. 2010). This result was a major step forward that provides a springboard for future development. One area that needs improvement is the immunization regime. First, all of the mice were initially primed by subcutaneous injection using soluble protein fractions that were enriched for CTB fusions. Second, ground up leaf material was prepared under liquid nitrogen and stored at −80 °C. Whether or not cold storage was necessary is unclear. Freeze-dried leaf material can often be stored at ambient temperatures while maintaining antigen integrity. Even if cold storage of leaf material is not necessary, purification and injection negatively impact vaccine cost and ease of administration. Importantly, this study used stable transgenic chloroplasts as opposed to transient expression through *Agrobacterium*-mediated transformation (Davoodi-Semiromi et al. 2010). Despite being the best choice for high protein yields, transient expression from *Agrobacterium* transformation is not ideal for oral vaccines because of potentially harmful contaminants that must be removed.

There are only two examples of alga-based oral vaccines for malaria. The first looked at AMA1 and MSP1 from the rodent malaria, *Plasmodium berghei* (Dauvillee et al. 2010). Rodent malaria provides a safe, convenient model system for studying the entire parasite lifecycle and vaccine efficacy because of its similarity to human malaria. MSP1 or AMA1 peptides were fused to the granule bound starch synthase (GBSS), expressed from the *C. reinhardtii* nuclear genome, and successfully targeted to starch granules. Mice that were orally vaccinated with LTB and purified starch granules containing GBSS-MSP1and GBSS-AMA1 showed a delay in

mortality and reduced parasitemia after a lethal challenge of *P. berghei* that was comparable to intraperitoneal (IP) injection. Unfortunately, the dose of antigen in starch granules is unclear, and the function of antibodies from oral vaccination was not tested further. It would be interesting to compare the inhibitory activity of antibodies from oral and IP vaccination in red blood cell invasion assays, which is commonly done to assess the efficacy of blood stage vaccine candidates. Nevertheless, this strategy warrants further investigation because specialized microfold cells (M cells) found in Peyer's patches (PP), or organized bundles of lymphoid tissue found in the ileum that are primary sites for adaptive immune induction, preferentially take up particulate matter. Starch granules, if they remain intact with antigen in the gut, may facilitate an adaptive immune response via PPs.

We investigated a simpler strategy utilizing whole *C. reinhardtii* cells. CTB-Pfs25 was produced as a fusion protein in *C. reinhardtii* chloroplasts and orally delivered to balb/c mice in freeze-dried whole cells (Gregory et al. 2013). This strategy elicited CTB specific IgG antibodies and secretory IgA antibodies to both CTB and Pfs25. Pfs25-specific serum IgG antibodies that can be taken up during a mosquito blood meal are necessary for transmission blocking activity. Thus, orally delivered algae containing CTB-Pfs25 is not a suitable TBV. An identical strategy examining the *Staphylococcus aureus* D2 fibronectin binding domain (D2) detected serum IgG and IgA antibodies to both CTB and D2 (Dreesen et al. 2010). There are two potential explanations for the discrepancy between the two studies. First, expression of CTB-D2 was significantly higher than CTB-Pfs25 (0.7 vs 0.09 %, respectively), so it could simply be a dosing issue. Second, D2 is inherently more immunogenic than Pfs25 (Brennan et al. 1999; Qian et al. 2007). Together, these studies suggest that the immune response to oral vaccines is, at least in part, dependent on the antigen itself. It is conceivable that alternative mucosal adjuvants might elicit different immune responses. In the case of Pfs25, an adjuvant that facilitates a Pfs25 specific IgG response would be more appropriate than the IgA only response elicited by CTB-Pfs25. Identifying and characterizing mucosal adjuvants is therefore critical to the success of oral vaccines.

Exploring mucosal adjuvants

Cholera toxin (CT) and the *E. coli* heat-labile toxin (LT) are arguably the best-characterized mucosal adjuvants to date(Sanchez and Holmgren 2011). They consist of a catalytically active α subunit (CTA and LTA) and a targeting β subunit pentamer (CTB and LTB), which binds GM1 ganglioside receptors on gut epithelial cells. Ultimately, CT causes massive fluid loss in the intestine through ADP-ribosylation of G proteins by CTA (Sanchez and Holmgren 2011). Removing CTA, and its associated toxicity, and replacing it with antigen provide a convenient targeting mechanism. Conjugation to CTB facilitates transport across the mucosal barrier and increases antigen uptake by antigen presenting cells (APCs). Despite extensive investigation of CTB, the immunomodulatory activity of CTB is still debated. Some studies suggest that it promotes immunity (Holmgren et al. 2005; Miyata et al. 2012), while others find that it promotes tolerance (Stanford et al. 2004; Sun et al. 2013). This contrast exemplifies our lack of understanding of how mucosal adjuvants facilitate an immune response to fused antigens. It is likely that the combined immunomodulatory effect of both the mucosal adjuvant and the fused antigen determines the overall outcome, which might be different depending on the immunization regime, the vehicle used for oral delivery, and the immune cells that are responsible for mediating the immune response. Our understanding of the interplay between these factors is currently limited for most mucosal adjuvants.

Mechanistic studies of potential mucosal adjuvants may eventually allow oral vaccines to be tailored to specific pathogens. For example, pre-erythrocytic malaria vaccines require

Table 1 Potential mucosal adjuvants for oral vaccines

	Examples (source)	Mechanism of action
Facilitated uptake	Cholera toxin B (*Vibrio cholerae*)	Binding to GM1
	Heat-labile enterotoxin B (*E. coli*)	Binding to GM1
	Sigma 1 (Reovirus) (Rynda et al. 2008)	M cell ligand
	Invasin (*Yersinia* (Autenrieth and Autenrieth 2008) and *Shigella* (Kaminski et al. 2006))	M cell ligand
	Co1 (12 a.a. peptide identified from library Screen (Kim et al. 2010))	M cell ligand
Pathogen-associated molecular patterns	Flagellins (*S. typhimurium*, *Listeria*, and others) (Mizel and Bates 2010)	TLR5
	Profilin (*Toxoplasma gondi* (Yarovinsky et al. 2005))	TLR11
Cytokines	IL-12(Vignali and Kuchroo 2012)	Th1
	GM-CSF(Zhan et al. 2012)	Maturation of macrophages and DCs

both circulating IgG antibodies and cytotoxic T cells while transmission blocking vaccines require only circulating IgG antibodies. Secretory IgA antibodies at mucosal surfaces, while less important for malaria, can help to mediate protection from gastrointestinal (polio, *typhoid*, *E. coli*), genital (HIV, HPV), and respiratory (*Mycobacterium tuberculosis*, influenza virus) pathogens. Importantly, systemic immunization is particularly poor at activating mucosal immunity compared to oral and intranasal routes of immunization (Cerutti 2008).

There are several proposed mucosal adjuvants other than CT and LT that may amplify an adaptive immune response (partial list in Table 1). These proteins act at different stages of the cascade of events that usually occur during a natural gastrointestinal infection. The first step is antigen introduction to the GALT. M cells are a primary port of entry to the GALT for many pathogens and are a proposed site of immune induction (Yamamoto et al. 2012). The hypothesis is that targeting to M cells more efficiently delivers antigen to APCs, which can then migrate to the mesenteric lymph node and induce an adaptive immune response through B and T cells. However, M cell ligands have been shown to induce tolerance and immunity depending on the ligand, antigen, and method of delivery, suggesting that targeting alone is not sufficient. Pathogen associated molecular pattern (PAMP) proteins induce cytokine production through TLRs, which impacts antigen presentation by DCs. For example, Flagellin is a potent activator of TLR5 and induces a mixed Th1 and Th2 immune response. TLR5 is particularly relevant to oral vaccines because it is highly expressed on gut DCs and resident macrophages (Uematsu and Akira 2009). Orally delivered plant tissue containing ovalbumin (OVA), the main protein found in egg white, fused to Flagellin, increased cellular and humoral immunity to OVA compared to OVA alone (Girard et al. 2011). Using specific cytokines rather than relying on induction by PAMPs has also been proposed (Tovey and Lallemand 2010). Deconvolving the relative adjuvanticity will require side-by-side comparisons using the same antigen, dosing, and delivery method. A particularly attractive approach would be to combine M cell targeting with TLR agonists. This would ensure that antigen and adjuvant were delivered to APCs.

Concluding remarks

Malaria has the greatest impact on many of the poorest regions of the globe. Vaccines are the most cost effective means for disease prevention and will be the key to eradicating malaria, but expensive injectable vaccines that require cold storage are not a practical option. Production of vaccines in plants and algae can drastically reduce costs, and making these vaccines orally available will further reduce costs and make administration easier if the many obstacles to oral delivery can be overcome. Future development of oral vaccines will depend on a better understanding of the immune response to orally delivered proteins and adjuvants.

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