Chloroplast-Derived Vaccine Antigens Confer Dual Immunity Against Cholera and Malaria by Oral or Injectable Delivery

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Abstract

Cholera and malaria are major diseases causing high mortality. The only licensed cholera vaccine is expensive; immunity is lost in children within 3 years and adults are not fully protected. No vaccine is yet available for malaria. Therefore, in this study, the cholera toxin-B subunit (CTB) of *Vibrio cholerae* fused to malarial vaccine antigens apical membrane antigen-1 (AMA1) and merozoite surface protein-1 (MSP1) was expressed in lettuce and tobacco chloroplasts. Southern blot analysis confirmed homoplasmy and stable integration of transgenes. CTB-AMA1 and CTB-MSP1 fusion proteins accumulated up to 13.17% and 10.11% (total soluble protein, TSP) in tobacco and up to 7.3% and 6.1% (TSP) in lettuce respectively. Nine groups of mice (*n* = 10/group) were immunized subcutaneously (SQV) or orally (ORV) with purified antigens or transplastomic tobacco leaves. Significant levels of antigen-specific antibody titres of immunized mice completely inhibited proliferation of the malarial parasite and cross-reacted with the native parasite proteins in immunoblots and immunofluorescence studies. Protection against cholera toxin challenge in both ORV (100%) and SQV (89%) mice correlated with CTB-specific titres of intestinal, serum IgA and IgG1 in ORV and only IgG1 in SQV mice, but no other immunoglobulin. Increasing numbers of interleukin-10+ T cell but not Foxp3+ regulatory T cells, suppression of interferon-γ and absence of interleukin-17 were observed in protected mice, suggesting that immunity is conferred via the Tr1/Th2 immune response. Dual immunity against two major infectious diseases provided by chloroplast-derived vaccine antigens for long-term (>300 days, 50% of mouse life span) offers a realistic platform for low cost vaccines and insight into mucosal and systemic immunity.

Keywords

cholera, malaria, chloroplast, vaccine, oral delivery, lettuce

Disciplines

Dentistry

Comments

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Summary

Cholera and malaria are major diseases causing high mortality. The only licensed cholera vaccine is expensive; immunity is lost in children within 3 years and adults are not fully protected. No vaccine is yet available for malaria. Therefore, in this study, the cholera toxin-B subunit (CTB) of *Vibrio cholerae* fused to malarial vaccine antigens apical membrane antigen-1 (AMA1) and merozoite surface protein-1 (MSP1) was expressed in lettuce and tobacco chloroplasts. Southern blot analysis confirmed homoplasmy and stable integration of transgenes. CTB-AMA1 and CTB-MSP1 fusion proteins accumulated up to 13.17% and 10.11% (total soluble protein, TSP) in tobacco and up to 7.3% and 6.1% (TSP) in lettuce respectively. Nine groups of mice (*n* = 10/group) were immunized subcutaneously (SQV) or orally (ORV) with purified antigens or transplastomic tobacco leaves. Significant levels of antigen-specific antibody titres of immunized mice completely inhibited proliferation of the malarial parasite and cross-reacted with the native parasite proteins in immunoblots and immunofluorescence studies. Protection against cholera toxin challenge in both ORV (100%) and SQV (89%) mice correlated with CTB-specific titres of intestinal, serum IgA and IgG1 in ORV and only IgG1 in SQV mice, but no other immunoglobulin. Increasing numbers of interleukin-10[+] T cell but not Foxp3^{+} regulatory T cells, suppression of interferon-γ and absence of interleukin-17 were observed in protected mice, suggesting that immunity is conferred via the Tr1/Th2 immune response. Dual immunity against two major infectious diseases provided by chloroplast-derived vaccine antigens for long-term (>300 days, 50% of mouse life span) offers a realistic platform for low cost vaccines and insight into mucosal and systemic immunity.

Introduction

Cholera is one among the top three diseases listed by the World Health Organization and the mortality rate is estimated to be 100 000–150 000 deaths annually (Longini *et al.*, 2007). Rapidly waning immunity with infection both from human and environmental sources has been recently reported (King *et al.*, 2008). However, only one internationally licensed cholera vaccine is available but this remains prohibitively expensive for routine use in cholera-endemic areas in developing countries (Mahalanabis *et al.*, 2008), especially at times of outbreak. Also, with the current cholera vaccine, immunity is lost in children within 3 years and adults are not fully protected (Olsson and Parment, 2006) suggesting the need for durable and low cost

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cholera vaccine. *Vibrio cholerae* secretes a 86-kDa toxin that is made up of two subunits: an α- and a β-subunit (CTB) that contains binding site for the plasma membrane receptor of the intestinal epithelial cells (GM1; de Haan *et al.*, 1998; Tsuji *et al.*, 1995) and this is an ideal antigen candidate for oral vaccine (Tacket *et al.*,1998,2004).

Malaria is also a devastating global health problem in tropical and subtropical areas of over 100 countries. *Plasmodium falciparum* is the most virulent species with approximately 500 million cases, 1 million deaths annually and more than 2 billion people are at risk for malaria (Greenwood *et al.*, 2005; Langhorne *et al.*, 2008). There are many challenges in developing a vaccine against malaria because of the complexity of antigens, high polymorphism among parasitic proteins, lack of appropriate animal models, high cost of vaccine development and delivery (Aide *et al.*, 2007). Currently, there is no licensed vaccine for prevention of malaria. Current clinical trials are under way investigating several blood-stage candidates such as apical membrane antigen-1 (AMA1), merozoite surface protein-1 (MSP1) and erythrocyte surface antigen (Greenwood *et al.*, 2005; Maher, 2008).

Mucosal- and gut-associated lymphatic tissues represent unique architecture of the immune system (Mora and von Andrian, 2008) and provide a major site of entry for many gastrointestinal, respiratory and urogenital infections, causing severe acute diseases (Holmgren and Czerkinsky, 2005; Rescigno and Chieppa, 2005). This compartment of the immune system constitutes a first line defense by providing antigen-specific local IgA, systemic antigen-specific immunoglobulins and generation of cytotoxic T cells. Advantages of oral plant-based vaccines have been described previously (Daniell *et al.*, 2005; Holmgren and Czerkinsky, 2005; Davoodi-Semiromi *et al.*, 2009).

The skyrocketing expense of current vaccines can be attributed to their unnecessarily complex production and delivery methods—from the significant cost of fermentation systems to purification through the use of complex technologies and additional expenses associated with adjuvant, cold storage, transportation and sterile delivery. This unconventional concept uses freeze-dried plant cells for bio-encapsulation of vaccine antigens that are protected in the stomach from acids and enzymes but are released to the immune system in the gut when plant cell walls are digested by bacteria that colonize the gut (Limaye *et al.*, 2006; Arlen *et al.*, 2008). Several vaccine antigens have been produced in nuclear transgenic plants for oral delivery against bacterial pathogens (Brodzik *et al.*, 2009) and shown to elicit immunoglobulin response and protection upon oral delivery in humans (Tacket *et al.*, 1998, 2004) and in mice (Arakawa *et al.*, 1998; Nochi *et al.*, 2007). Also, plant-derived recombinant F1, V and F1-V fusion antigens of *Yersinia pestis* protected guinea pigs against an aerosol challenge of virulent *Y. pestis* (Del Prete *et al.*, 2009). One of the major limitations has been the ability to accumulate sufficient levels of protein, either for purification or for oral delivery in minimally processed plant tissues. The integration of transgenes via the nuclear genome may have other disadvantages including transgene containment, gene silencing and position effect. Moreover, no transgenic plant-based vaccine has moved beyond phase I clinical trial (Yusibov and Rabindran, 2008), highlighting the need to explore new technologies.

The chloroplast genetic engineering approach can address concerns on transgene containment (Daniell, 2002). Genetically modified chloroplast genomes of most crops are maternally inherited and the absence of any reproductive structures offer efficient foreign gene containment and facilitate their safe production in the field (Daniell, 2007). It also overcomes the concerns of gene silencing and position effect (De Cosa *et al.*, 2001; Lee *et al.*, 2003) and pleiotropic effects (Lee *et al.*,2003; Ruiz *et al.*, 2003). Multigene engineering is possible with chloroplast transformation because of its prokaryotic nature (Grevich and Daniell, 2005; Quesada-Vargas *et al.*, 2005). In addition, chloroplasts are highly efficient bioreactors (Daniell *et al.*, 2002, 2004; Daniell, 2006; Verma *et al.*, 2008; Davoodi-Semiromi *et al.*, 2009) and it

is possible to produce up to 360 million doses of fully functional anthrax vaccine in one acre of tobacco (Koya *et al.*, 2005). Several therapeutic proteins and vaccine antigens have been expressed via the chloroplast genome against bacterial, viral and protozoan pathogens, including the CTB (Daniell *et al.*, 2001), heat labile toxin-B subunit (Rosales-Mendoza *et al.*, 2009), anthrax protective antigen (Watson *et al.*, 2004; Koya *et al.*, 2005), plague F1-V (Arlen *et al.*, 2008), tetanus toxin (Tregoning *et al.*, 2003, 2005), vaccinia virus (Rigano *et al.*, 2009), the 2L21 peptide from the canine parvovirus (Molina *et al.*, 2004, 2005), HEV E2 of Hepatitis E virus (Zhou *et al.*, 2006) and the GAL/GALNAc lectin of *Entamoeba histolytica* (Chebolu and Daniell, 2007). These vaccine antigens expressed in transgenic chloroplasts have proper post-translational modifications and are fully functional by appropriate immune response in animal models and/or protection conferred against pathogen or toxin challenge. Oral delivery of plant cells producing human proinsulin in chloroplasts prevented the onset of type 1 diabetes in non-obese diabetic mice (Ruhlman *et al.*, 2007). Oral immunization (ORV) of chloroplast-derived F1-V antigens without adjuvant conferred greater protection (88%) against 50-fold lethal dose of aerosolized plague (*Yersinia pestis*) than subcutaneous (SQV) immunization (33%) (Arlen *et al.*, 2008). Optimal protection against diseases upon oral delivery of vaccine antigens demonstrate that chloroplast can produce fully functional antigens, survive in the stomach by bioencapsulation and when released in the gut, make vaccine more efficacious than the same protein delivered through the perenteral route.

However, one among the major limitations in this field is the expression of several therapeutic proteins in tobacco, which is not suitable for oral delivery. To avoid prohibitively expensive purification, cold storage/transportation and achieve oral delivery, optimal expression of therapeutic proteins in chloroplasts of edible crops is necessary. We developed the carrot plastid transformation system with adequate levels of gene expression in the edible parts to facilitate oral delivery (Kumar *et al.*, 2004); however, the regeneration is extremely slow and it is not suitable for rapid production of therapeutic proteins. Although high level expression of human immunodeficiency virus p24 antigen was observed in tomato leaves, there was a >90% reduction in green fruits and no expression was observed in red ripe fruits (Zhou *et al.*, 2008). The lettuce chloroplast transformation has been developed by several groups (Lelivelt *et al.*, 2005; Kanamoto *et al.*, 2006) but expression of therapeutic proteins was unsuccessful. We have optimized the lettuce plastid transformation system and in this study attempt to express vaccine antigens. The level of expression in lettuce chloroplast is similar to tobacco and regeneration is as rapid as tobacco, the most successful plastid transformation system developed so far.

In this study, we conjugate CTB with two major malarial vaccine antigens, AMA1 and MSP1, in tobacco and lettuce chloroplasts and female BALB/c mice were immunized orally with tobacco transplastomic leaves or subcutaneously with purified or enriched vaccine antigens. Different groups of immunized mice and control were challenged with cholera toxin or sera were evaluated against the malarial parasite. Oral immunization provided both mucosal and systemic immunity while subcutaneously immunized mice developed systemic immunity. Increasing numbers of interleukin (IL)-10$^+$CD4$^+$ T cell along with higher concentration of antigen-specific IgG1 suggests Tr1/Th2 type immunity was generated in immunized mice. Numbers of Foxp3+ regulatory T cells, IL-17 and interferon (IFN)-γ do not play any significant role in protection against cholera toxin challenge. Sera of mice immunized with the malarial vaccine antigens markedly blocked proliferation of the malarial parasite in red blood cells (RBC), confirming efficacy and specificity of generated antibody against the malarial parasite. This study provides insight into the longevity of mucosal and systemic immunity and confirms that durable immunity can be maintained in older mice with additional boosters. High level of protection conferred by chloroplast-derived CTB conjugated with the malaria vaccine antigens should facilitate development of a low cost multivalent vaccine for large populations, at times of outbreak.

Results

Characterization of lettuce and tobacco transplastomic lines expressing vaccine antigens

Tobacco chloroplast vectors contained the *trn*I (Ile) and *trn*A (Ala) genes while lettuce chloroplast vectors contained 16S/*trn*I and *trn*A/23S genes as flanking sequences (longer flanking sequence than tobacco) for homologous recombination with the native chloroplast genome (Ruhlman *et al.*, 2007). Expression cassettes for vaccine antigens CTB-AMA1 and CTB-MSP1 were regulated by endogenous *psb*A promoter, 5′-untranslated region (UTR) to enhance translation and the 3′-UTR to confer transcript stability (Figure 1). CTB-AMA1 fusion included the GPGP (Gly Pro Gly Pro) hinge region and the furin cleavage site while CTB-MSP1 included only the GPGP hinge in between fusion proteins to facilitate correct folding of each protein by reducing the steric hindrance. The native plastid ribosomal operon promoter (P*rrn*) was used to drive expression of the *aad*A gene from the GGAG ribosome binding site for spectinomycin resistance. After completion of chloroplast vectors pLD CTB-AMA1, pLD CTB-MSP1, pLsDV CTB- and pLsDV CTB-MSP1 (Figure 1), plasmids were isolated, sequenced and used for chloroplast transformation studies.

Transplastomic tobacco and lettuce plants were obtained as described previously (Kanamoto *et al.*, 2006; Ruhlman *et al.*, 2007; Verma *et al.*, 2008). Five to six primary tobacco and 3–6 lettuce transformants (per 10 bombardments) appeared 3–6 weeks after bombardment from leaves placed on the regeneration medium containing the selection agent. Duration of lettuce regeneration and efficiency of transformation is very similar to tobacco. Primary transformants were screened by polymerase chain reaction (PCR) using 3P/3M and 5P/2M primer pairs in tobacco and 16SF/3M and 5P/2M primer pairs in lettuce (data not shown). Following an additional round of selective regeneration, progenitors for each transplastomic line was rooted in medium containing the selection agent. Clones were transferred to Jiffy® peat pots, acclimatized in biodome and moved to the greenhouse, where they matured, flowered and produced seeds.

Southern blot analysis confirmed site specific transgene integration into the chloroplast genome and homoplasmy in all tobacco and lettuce transgenic lines (Figure 2). Tobacco CTB-AMA1 and CTB-MSP1 transplastomic lines yielded 6.6- and 6.5-kb fragment respectively while untransformed line yielded a 4.1-kb fragment (Figure 2a). Lettuce transplastomic lines with CTB-AMA1 yielded 11.6 kb and CTB-MSP1 yielded 11.5 kb, while untransformed lines yielded 9.1-kb fragment (Figure 2b). The absence of untransformed fragment in lettuce and tobacco transplastomic lines confirmed that they achieved homoplasmy. Presence of the CTB-coding sequence in transplastomes was confirmed by the CTB probe (Figure 2c).

Expression, quantification and enrichment of vaccine antigens

Immunoblots were performed with tobacco and lettuce transplastomic lines expressing CTB-AMA1 and CTB-MSP1 (Figures 3a–d). Immunodetection with CTB polyclonal antibody showed 11.5 kDa of the CTB monomer, 27.5 kDa monomer of CTB fused with AMA1 and a 23 kDa monomer of CTB fused with MSP1 (Figures 3a–d). The formation of dimers, trimers, tetramers and pentamers of CTB-AMA1 and CTB-MSP1 fusion proteins was observed in tobacco as well as in lettuce. Foreign proteins could be detected in the supernatant and pellet (Figures 3a,b). Therefore, the quantification of CTB-AMA1 and CTB-MSP1 was performed using homogenate.

The CTB-AMA1 expression level of tobacco T0 and T1 transplastomic lines in mature leaves reached up to 13.17% and 12.41% of the TSP respectively. The CTB-MSP1 expression level of tobacco T0 and T1 transplastomic lines in mature leaves reached up to 10.11% and 9.9% of the TSP respectively. In lettuce T0 and T1, CTB-AMA1 expression level reached up to 7.3%

and 7.26% of the TSP, respectively in mature leaves under the green-house growth conditions. In lettuce T0 and T1, CTB-MSP1 expression level reached up to 6.1% and 5.89% of the TSP, respectively in mature leaves under the green-house growth conditions. A gram of mature leaf contained up to 3.56 mg or 1.21 mg of CTB-AMA1 fusion protein in tobacco or lettuce respectively. A gram of mature leaf contained up to 2.72 or 0.83 mg of CTB-MSP1 antigen in transformed tobacco or lettuce respectively.

To determine the affinity of plant-derived CTB fusion protein for the GM1-ganglioside receptor, GM1-enzyme-linked-immunosorbent serologic assay (ELISA) was performed. GM_1-ganglioside has been shown to be the receptor for CTB protein *in vivo* (de Haan *et al.*, 1998) and a pentameric structure is required for binding to GM_1 receptor (Tsuji *et al.*, 1995). To address whether CTB fusion protein generated in transplastomic tobacco and lettuce retained biological activities and could bind to the GM_1 receptor, we performed GM_1-binding ELISA assay. As illustrated in Figure 3e, transplastomic CTB-AMA1 and CTB-MSP1 protein are fully functional and binds to GM_1. These results suggest that the tobacco and lettuce chloroplast-derived CTB-AMA1 and CTB-MSP1 assemble properly to form pentameric structures, which are essential for GM_1-ganglioside receptor binding.

Cholera toxin was shown to bind to immobilized Ni^{2+} ion mediated by the B subunit and is a useful affinity purification method for CTB and complexes composed of CTB (Dertzbaugh and Cox, 1998). Therefore, for enrichment crude plant extract was subjected to immobilized metal affinity chromatography using the TALON Superflow Metal Affinity Resin (Clontech) and run on NuPAGE Novex Bis– Tris gradient gel under reducing and non-reducing conditions. The large subunit of Rubisco (55 kDa) was present in the untransformed, lysate and flow through fractions under reduced and non-reduced conditions (Figure 4a). In the wash fractions, minimal number of proteins was observed. In the eluted CTB-AMA1 fraction, the monomer of 27.5 kDa in size was present under reduced conditions (lane 6) and the pentameric form was present under both reduced (lane 6) and non-reduced (lane 12) conditions (Figure 4a). It should be noted that the pentameric form was the dominant form and this should facilitate GM1 binding. An immunoblot probed with anti-CTB antibody was conducted to evaluate the CTB-malarial proteins after talon enrichment. An immunoblot with known concentrations of CTB protein and different concentrations of the enriched fractions was probed with anti-CTB antibody. Quantitation of the enriched CTB-malarial proteins on immunoblots was analysed by densitometry. Linearity of the standard curve assisted in the estimation of the enriched samples in the same blot (Figure 4b,c). The efficiency of the talon enrichment was determined to be 90% and 73% in CTB-AMA1 and CTB-MSP1 respectively.

Tobacco transplastomic lines were available before lettuce and therefore animal studies were performed with tobacco. Because presence of nicotine or other alkaloids has been a disadvantage for human oral delivery studies, it is important to create lettuce lines. Lettuce leaves are consumed without cooking by humans and the time from sowing seed to obtain biomass is only few weeks when compared with few months for other crops (such as tomato, potato and carrot). Therefore, lettuce lines expressing CTB-AMA1 and CTB-MSP1 were developed.

Humoral immunity in vaccinated mice confers protection against cholera toxin challenge

The immunization schedule is shown in Figure 5a. Control and immunized BALB/c mice were challenged with cholera toxin for 14 h. Then mice were euthanized and intestinal content was collected. A significant ($P < 0.0001$) correlation was observed between volume of intestinal water retention in SQV and ORV mice and protection (Figure 5b). There was no significant difference between these two groups (Figure 5b). All of control mice (100%), adjuvant (AJV) and/or immunized mice with untransformed leaf materials were not protected (Figure 5b,c). To explore impact of CT challenge on immunized/control mice, we screened presence/absence

of antigen-specific antibody in the sera. Antigen-specific ELISA data showed the presence of antigen-specific CTB-IgA in sera and intestinal content of ORV-CTB mice but not in any other group of mice tested, suggesting a direct correlation between IgA production and oral immunization (Figure 5c). It should be noted that IgA titres repeatedly and reproducibly observed in ORV-CTB mice in this study were much higher than those reported in previous studies (Arlen *et al.*, 2008).

In contrast, in SQV mice that were protected from CT challenge, we were unable to detect any CTB-IgA in sera and/or in intestinal content by ELISA. To investigate the mechanism of protection observed in SQV mice, we screened a broad range of antigen-specific immunoglobulins by ELISA including -IgG1, -IgG2a, -IgG2b, -IgG3 and -IgM using sera of vaccinated and control mice. As shown in Figure 6a, our data show that only CTB-IgG1 but not any other tested immunoglobulin conferred protection in SQV mice (Figure 6a, top row). Screening of the same profile of immunoglobulins in the sera of ORV mice showed comparable pattern of expression with SQV mice (Figure 6a) suggesting superiority of oral vaccination because orally immunized mice not only generated systemic antibodies like the other groups but in addition generated mucosal immunity, while the other groups failed to do so. These data suggest that immune responses to the same antigen might be different and this is dependent on the route of immunization. Furthermore, we screened additional antigen-specific antibodies including CTB-IgG1, -IgG2a, -IgG2b, -IgG3 and -IgM in the sera of vaccinated mice before (bleed # 5) and after CT challenge. Our data suggest that only antigen-specific CTB-IgM antibody level significantly changed after CT challenge but not any other antibodies tested (Figure 6a, bottom row).

To understand whether CT challenge might modulate the immune responses towards the Th1 (T-helper-1), Th2 (T-helper-2) and/or Th17 in immunized/control mice, we further screened expression of IL-4 (Th2), IL-10 (Th2), IL-2 (Th1), IFNγ (Th1) and IL-17A (Th17) by ELISA in the sera. Our data show that expression of IFNγ was detected in 70% (seven of 10 mice), 16.6% (one of six mouse) and 10% (one of 10 mice) of control, SQV and ORV-CTB mice, respectively suggesting that immunization blocks Th1 immune response. However, IL-17A is unlikely to play a role in this system because only 11% in SQV (one in nine) and 10% (one in 10 mice) in ORV-CTB groups were positive for IL-17 when examined by ELISA.

Kinetics of antigen-specific IgA in sera obtained from orally immunized mice showed an increase in antibody titre associated with the number of boosters. This data also showed that about five oral boosts are required to generate high levels of immunity (Figure 6b). There was significant variation in immune titres within the same group, even though BALB/c is an inbred strain. However, all animals were protected after toxin challenge in this group, showing that high levels of immune titres are probably not required for protection against the pathogen. Based on this data, to prolong immunity, additional boosters may be desirable but not required.

Protected mice had higher expression of interleukin-10$^+$CD4$^+$ T cells but not Foxp3$^+$CD4$^+$ T cells

Further investigations were performed to understand cellular immunity. Expression of different markers associated with T cells and antigen-presenting dendritic cells (DC) in freshly prepared splenocytes obtained from controls (unvaccinated) and vaccinated mice after CT challenge was measured. As shown in Figure 7a, CT challenge eliminated CD4$^+$IL10$^+$ T cells in unvaccinated control mice after CT challenge but CD4$^+$IL10$^+$ T cells markedly ameliorated in this population in SQV and ORV-CTB mice suggesting IL-10 expressing CD4 T cells (Tr1 regulatory T cells) may play a protective role in adaptive immunity generated by immunization. However, CT challenge did not increase numbers of CD4$^+$CD25$^+$ T cells population in control mice after CT challenge but moderately increased this population on SQV and ORV mice when compared with control mice. Intriguingly, CT challenge dramatically ameliorated numbers of

$CD4^+Foxp3^+$ regulatory T cell in unvaccinated mice after CT challenge (~2.5-fold) and this effect was moderate in SQV (ranged from 7.2% to 12.5%) and in ORV-CTB mice (range from 11.5% to 14%) (Figure 7b, middle row). In contrast, as shown in Figure 7b, CT decreased expression of IL7Rα (CD127) in unvaccinated mice after CT challenge but had marked up-regulation in SQV and ORV-CTB mice (~5%–8%). Based on these data, vaccinated mice with chloroplast-derived antigen generated a Tr1 cellular immune response and a Th2 humoral immune response that correlated with protection against the toxin challenge.

Impact of cholera toxin challenge on dendritic cells maturation in immunized mice

We further investigated the role of DC in cellular immunity generated in immunized mice. We evaluated expression of major histocompatibility complex (MHC) II, CD40 and CD80 on CD11c+ DC population, prepared from fresh splenocytes of 2–5 pooled mice. As shown in Figure 7c, expression of MHC II and co-stimulatory molecules in CD11c+ DC in SQV and ORV mice was almost similar to control unchallenged mice. However, expression of these markers in control (challenged) mice increased to about 8%–11% when compared with control (unchallenged) and ORV/SQV mice. Steady-state expression of CD11c and co-stimulatory molecule CD80 in immunized mice was very similar to the phenotype observed in tolerogenic DC. CT challenge had no or very little effect on maturation of splenic DC in vaccinated mice. However, CT increased expression of these markers in the control/challenged mice suggesting that immunization minimized the effect of CT on maturation of DC but not in control mice challenged with CT.

Antibodies generated in immunized mice cross-reacted with the *Plasmodium* antigens

Our data show increasing numbers of boosters with the malarial vaccine antigens increased the anti-MSP1 antibody titres in immunized mice (Figure 8a). Generation of anti-MSP1 antibody (IgG1) in SQV was higher than ORV (Figure 8a). As we showed earlier for CTB, ORV mice generated both systemic and mucosal immune responses for malarial antigens. However, SQV mice failed to generate IgA and this may account for lower anti-MSP1 IgG1 antibody titres in ORV mice. Limited quantity of AMA1 antigen provided by NIH was a major hurdle in this study. Therefore, we measured anti-AMA1 antibody in sera of immunized mice only in few experiments. To assess specificity of anti-plasmodium antibody in sera of mice immunized with the malarial vaccine antigens, we performed a series of immunoblots and immunofluorescence studies. Immunoblot data showed that anti-AMA1 antibody in the sera (bleed 4) of immunized mice hybridized and recognized schizont protein of *Plasmodium* as a 83-kDa polypeptide (Figure 8b, lane 3). The sera from immunized mice contained anti-MSP1 antibodies, also bound and recognized both ring and schizont proteins of *Plasmodium* as a 190-kDa polypeptide in immunoblot (Figure 8b, lane 4). Furthermore, anti-AMA1 antibodies obtained from sera of immunized mice successfully hybridized with the apical end of the parasite at the ring stage as shown in Figure 8c, further confirming specificity of antibody generated in immunized mice with chloroplast-derived vaccine antigen. As shown in Figure 8c, fluorescence-labelled sera from immunized mice with the chloroplast-derived CTB-MSP1 antigen successfully stained schizonts stage of malarial parasite further confirming specificity of antibody generated in vaccinated mice.

Antibodies generated in immunized mice blocks *Plasmodium* entry into red blood cells

To examine functionality of antibody generated in immunized mice against *Plasmodium*, parasite inhibition assays evaluated the ability of anti-MSP1 antibodies in inhibiting parasite entry into erythrocytes. We found that the ring stage was the predominant stage of the parasite under microscopic examination. Average parasitemia was determined to be in the range of 5.7%–6.6% in the blank control samples (no serum added), in untreated control animals and those gavaged with WT (untransformed) plants (Table 1). The lowest parasitemia was found

to be associated with MSP-119 group (group 5, s.c MSP1) that had the highest mean antibody titre (Figure 8a). Erythrocytes were protected 100% from parasite entry when they were treated with serum from positive control (MRA-35 rabbit antiserum purified from recombinant yeast, *Pf*MSP1–19, 3D7) and successful inhibition was observed when sera of immunized mice was used to block parasite entry into erythrocytes. However, sera of control mice failed to prevent parasite entry (Table 1). In this study, relative inhibition rate regardless of the route of immunization was 86%–117% ($\pm$15.5%). Relative inhibition of *Plasmodium* with the sera obtained from vaccinated mice (bleed # 4) was as efficient as or better than the positive serum used in this study (Table 1). Both oral and injectable vaccination with AMA-1 conferred 102% or 105% inhibition in parasitemia assays. Inhibition was slightly less when both antigens were delivered orally or by injection because 50% less antigen dose was delivered when compared with single antigens.

Discussion

Currently, there is no licensed vaccine for the prevention of malarial disease despite the vast knowledge of genomics and proteomics of the malaria parasite (Sharma and Pathak, 2008). Recently, phase 2a trial of *Falciparum* malaria vaccines RTS,S/AS01B and RTS,S/AS02A in malaria-naive adults has been conducted (Kester *et al.*, 2009). The only available cholera vaccine is unaffordable to most people in developing countries as it is highly expensive (Kim *et al.*, 2008). The high cost of current vaccines is mainly because of the method of production using expensive fermentation, purification and delivery systems. To address this, several vaccine antigens have been expressed via the nuclear genome (Yusibov and Rabindran, 2008), which elicited immunogenic response, and conferred protection upon oral delivery (Arntzen, 2008). However, immunogenic response was often less than adequate because of insufficient levels of the antigens expressed via the nuclear genome (Streatfield *et al.*, 2001; Streatfield, 2007). This led to development of an alternate method using plant chloroplast as bioreactors to produce high levels of vaccine antigens facilitated by >10 000 copies of transgene in each single transformed plant cells (Daniell *et al.*, 2001).

This study is the first report of a vaccine antigen expression in an ideal oral delivery system in chloroplasts. Expression of the influenza virus haemagglutinin gene in lettuce chloroplasts could not be detected in both young and mature leaves by immunoblots (Lelivelt *et al.*, 2005). Also in this study, we report the first chloroplast-derived dual vaccines by multigene engineering for two major infectious diseases, cholera and malaria. The two of the leading asexual blood-stage malarial vaccine candidate genes, *AMA1* and *MSP1*, were cloned along with the transmucosal carrier, CTB, and were successfully expressed via the tobacco and lettuce chloroplast genomes. We used native *psb*A 5′-UTR regulatory elements in tobacco and lettuce to drive *CTB-AMA1* and *CTB-MSP1* as it was shown to enhance translation of more foreign protein than the genes regulated by bacteriophage T7 gene 10 translational elements (Dhingra *et al.*, 2004). The expression levels in mature leaves of CTB-AMA1 and CTB-MSP1 protein reached up to 13.17% and 10.11% of TSP respectively in tobacco, whereas in lettuce, expression levels of CTB-AMA1 and CTB MSP1 was 7.3% and 6.1% of TSP respectively. The expression levels of CTB-AMA1 in tobacco is comparable to 14.8% accumulation of F1-V (Arlen *et al.*, 2008), 14% of anthrax protective antigen (Koya *et al.*, 2005) and 16% of CTB-Pins in tobacco (Ruhlman *et al.*, 2007). In previous studies, expression of cholera vaccine antigen has been demonstrated in edible crop plants including carrot (Rosales-Mendoza *et al.*, 2008), potato (Arakawa *et al.*, 1997) and tomato (Jani *et al.*, 2002). Recently CTB protein has been targeted to endoplasmic reticulum in carrot but the expression level could reach up to 0.48% of TSP (Kim *et al.*, 2009). The expression level in these nuclear transgenic crop plants ranged from 0.02% to 0.48% of TSP. This low level of expression would require large amount of plant material for human immunization. In the present study, the CTB-AMA1 and CTB-MSP1, dual cholera-malaria vaccine antigens accumulated 100- to 600-fold higher in tobacco

and 100- to 400-fold higher in lettuce than nuclear expressed cholera vaccine antigens. The cholera-malaria vaccine antigens expressed in lettuce (6.1%–7.3% of TSP) is sufficient to perform animal and human clinical studies.

Production of a green vaccine for major infectious diseases such as cholera and malaria with ease of administration that does not require cold chain is an important need, especially in areas with limited access to cold storage or transportation. Considering that mucosal surface is the site for many gastrointestinal, respiratory and urogenital infections, developing an oral vaccine has great significance. For instance gastrointestinal infections caused by *V. cholerae*, *Helicobacter pylori*, *Shigella* spp. and/or by rotaviruses, *Entamoeba histolytica* are major examples among many others. Many advantages of oral plant-derived vaccines to confer immunity against aforementioned infectious agents were discussed previously (Daniell *et al.*, 2005; Holmgren and Czerkinsky, 2005; Davoodi-Semiromi *et al.*, 2009).

Our investigation is the longest cholera vaccine study reported so far in the plant-derived vaccine literature. Animals were boosted until 267 days and were challenged on day 303. Multiple oral boosters conferred durable immunity, more than half of the mouse (BALB/c) life span. This observation is important in the light of recent reports on waning immunity against cholera (King *et al.*, 2008). With the current cholera vaccine, immunity is lost in children within 3 years and adults are not fully protected (Olsson and Parment, 2006). Although boosters beyond 5–8 times did not significantly increase immunity levels, long-term protection was maintained. No significant statistical differences were observed in CTB-IgA in sera of immunized mice that received 5–8 shots when compared with antibody level of the same mice with more than eight shots.

In the current study, we observed high level of CTB-IgA only in ORV-CTB mice but not in SQV or AJV or ORV-UT mice. In contrast, antigen presentation to the mucosal immune system via a non-receptor-mediated delivery resulted in little or no local antigen-specific IgA (Arlen *et al.*, 2008). Immunization by i.p. with outer membrane vesicles of *V. cholera* failed to generate IgA (Schild *et al.*, 2008, 2009). These data suggest that induction of intestinal IgA may require certain components of the gut immune system which does not exist in any other part of the body. Further studies with antigens conjugated with and without CTB or other proteins that bind to intestinal receptors are necessary to understand the relationship between antigen presentation and production of IgA. Recently it has been shown that interaction of intestinal IgA with other locally generated cytokines such as transforming growth factor-β1, IL-10 and IL-4 will provide a unique microenvironment to educate DC and subsequently educated DC will imprint naïve T cells (Alpan *et al.*, 2004) and imprinted T cells secrete the same cytokine profile as previously antigen-experienced T cells. None of SQV mice had detectable CTB-IgA; however, 89% of SQV mice were protected from CT challenge. Our data show that only serum CTB-IgG1 and not -IgG2a, -IgG2b, -IgG3 or -IgM conferred immunity against CT challenge in SQV mice. Our data also show that only CTB-IgM significantly decreased after CT challenge, while other members of the family remained the same.

In our study, CT induced up-regulation of IL-10 expressing $CD4^+$ T cells, CTB-IgA and CTB-IgG1 in ORV and SQV, respectively, suggesting that vaccination regiment elicited Tr1/Th2 immune response and protected vaccinated mice against CT challenge. Up-regulation of IL-7Rα$^+$Foxp3$^-$CD4$^+$ T cell in vaccinated mice after CT challenge is interesting because it has been reported that formation of Peyer's patches is dependent upon IL-7 receptor, tumour necrosis factor (TNF) and TNF super family members (Fu and Chaplin, 1999). Further experiments are needed to address functional properties of IL-7R in plant-derived vaccines and immunity. Furthermore, our data from single-cell-based studies suggest that CT increased numbers of Foxp3$^+$ regulatory T cells and co-stimulatory molecule CD80 in splenocytes in unvaccinated control mice but CT had little effect on this population in vaccinated mice.

Increasing numbers of Foxp3 regulatory T cells in unvaccinated mice is interesting because this population is the most effective arm of peripheral tolerance. Immediate consequences of higher numbers of Foxp3$^+$ regulatory T cell would be suppression of responding T-cell populations to CT (Shevach, 2002). Because CT did not increase numbers of CD4$^+$CD25^{+high} T cells (data not shown), it appears that CT converts Foxp3$^-$CD25$^-$CD4$^+$ T cells into Foxp3$^+$ regulatory T cells in the periphery. In agreement with our data, Sun *et al.* (2006) have reported increasing number of Ag-specific Foxp3$^+$ regulatory T cells by CTB and CTB$^+$ CT respectively. They have also demonstrated that intragasteric administration of OVA-CTB-induced expression of antigen-specific Foxp3$^+$CD25$^+$ regulatory T cells.

Despite the recent increase in knowledge of genomics and proteomics of the malarial parasites, no licensed vaccine for the prevention of malarial disease is yet available (Sharma and Pathak, 2008). The need for a malarial vaccine is imperative because the global burden of the disease is increasing because of drug resistance, mosquito's resistance to insecticides, ineffective control measures, re-emergence of the disease and increased tourism. There is a great need to create a low cost human malarial vaccine with the elimination of laborious and expensive purification techniques. Two leading blood-stage malarial vaccine candidates, AMA1 and MSP1 were constructed in a fusion cassette with CTB. It has been reported that malarial antigens display poor immunogenicity even when used with adjuvants (Sachdeva *et al.*, 2006). Several strategies for increasing immunogenicity of malarial antigens include the use of different adjuvants, optimizing immunization protocols, using rabbits or monkeys for animal testing, fusing malarial antigen with viral or bacterial antigens, or constructing multivalent antigen chimeras have been reported (Wang *et al.*, 2003; Pan *et al.*, 2004; Qian *et al.*, 2007; Greenwood *et al.*, 2008). The expression of human malarial antigen, *Plasmodium falciparum* MCP1 COOH-terminal region in tobacco plants via the nuclear genome has been reported earlier (Ghosh *et al.*, 1993). Recently, the rodent malarial antigen, *P. yoelii* codon-optimized MSP 4/5 was expressed in tobacco transgenic plants (Wang *et al.*, 2008) that showed modest expression level (0.25% TSP). In our study, the human malarial antigens consisting of domain III of AMA1 and 19-kDa C-terminal fragment of MSP1 showed high levels of expression in both lettuce and tobacco chloroplasts (up to 13.17% TSP). Expression of AT-rich *P. falciparum* (Gardner *et al.*, 2002) open-reading frames is compatible with chloroplast expression system because the chloroplast genome is also AT-rich. The recombinant chimeric antigen was found to be highly immunogenic in mice. Our *in vitro* inhibition assay provided evidence that the antibodies generated from immunized mice were effective in preventing parasite invasion of RBC. Evaluation of human vaccine antigens (*P. falciparum*) in the rodent model system has major difficulties. Malaria challenge failed when mice are vaccinated with *P. falciparum* and challenged with *P. berghei* (de Koning-Ward *et al.*, 2003). One solution to this problem is to challenge immunized mice with the *P. berghei*/*P. falciparum* (Pb-PfM19) chimeric line that expresses the *P. falciparum* MSP1 (Thomas *et al.*, 1994). Parasite challenge was shown to be successful to protect mice when animals were passively immunized with anti-*Pf*MSP1$_{42}$ antibody and then challenged with chimeric line (Sachdeva *et al.*, 2006). However, in our study this chimeric *P. berghei* line was ineffective because administration of this strain did not cause malaria in unimmunized control mice.

In conclusion, this study for the first time demonstrates possibility of generation of multivalent green vaccine against two major infectious diseases, cholera and malaria. Currently, other than the rotavirus, there is no other example of oral vaccines in the US and the mucosal immune system has not been utilized to confer immunity against invading pathogens. Oral polio vaccine was discontinued in the US because one in 2.4 million cases contracted polio from the live attenuated oral vaccine. However, such problems are not associated with subunit vaccines because only one or two antigens are used that are incapable of causing any disease. Therefore, it is important to understand and utilize the mucosal immune system for delivery of subunit vaccines. Bioencapsulation of vaccine antigens in plant cells provide an ideal low cost delivery

system for large-scale distribution at times of crisis. In addition, oral delivery confers dual protection via systemic and mucosal immune system. High level and long-term protection observed against cholera toxin challenge and against the malarial parasite in mice sera immunized with chloroplast-derived antigens, makes this system yet another new platform for advancing towards human clinical studies.

Experimental procedures

Chloroplast vector construction and regeneration of transplastomic plants

The AMA1 and MSP1 were synthesized (Pan *et al.*, 2004) and cloned into the pGEMT Easy Vector (Promega). The CTB sequence was amplified using pLD-5′-UTR-CTB-Pins (Ruhlman *et al.*, 2007) vector as the template. Further, the sequences were verified to check any errors, fused and subcloned into the pBSSK+ (Stratagene) vector. CTB-AMA1 fusion had GPGP hinge region and the furin cleavage site while CTB-MSP1 only had the GPGP hinge in between fusion proteins to facilitate correct folding of each protein by reducing the steric hindrance. The CTB-AMA1 and CTB-MSP1 expression cassette was created with tobacco regulatory elements and ligated into the pLDctv tobacco chloroplast transformation vector to obtain pLD CTB-AMA1 and pLD CTB MSP1 (Daniell *et al.*, 1998, 2004). Leaves of *Nicotiana tabacum* var. Petite Havana were bombarded with pLD CTB-AMA1 and pLD CTB-MSP1 and the transformants were obtained as described earlier (Singh *et al.*, 2009).

The pUC-based *Lactuca sativa* long flanking plasmid sequence (pLSLF) was used to integrate foreign genes into the intergenic spacer region between the *trn*I (Ile) and *trn*A (Ala) genes as described previously (Ruhlman *et al.*, 2007; Verma *et al.*, 2008). The P*rrn: aad*A: *rbc*L selectable marker gene cassette was assembled in pBSSK+ vector and contained the *rrn* promoter and *rbc*L 3′-UTR amplified from the lettuce chloroplast genome. The *aad*A expression cassette was subcloned into pLSLF resulting in the pLsDV vector. The pLsDV CTB-AMA1 and pLsDV CTB-MSP1 were constructed using CTB-AMA1 and CTB-MSP1 expression cassette with the *psb*A promoter, 5′- and 3′-UTR from lettuce. All cloning steps to create chloroplast transformation vectors were performed in *Escherichia coli* using standard molecular biology protocols (Sambrook and Russell, 2001; Verma *et al.*, 2008). *Lactuca sativa* var. Simpson elite was transformed and the transplastomic lines were selected as described previously (Ruhlman *et al.*, 2007). PCR reactions were performed using two sets of primers namely 3P/3M or 16SF/3M and 5P/2M. Southern blot analysis was performed to confirm transgene integration as well as homoplasmy as described earlier (Singh *et al.*, 2009).

Immunoblot analysis and enrichment of chloroplast-derived proteins

Chloroplast-derived CTB-malarial proteins were extracted from 100 mg of powdered leaf material. Plant extraction buffer [200 μL; 100 mM NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA), 200 mM Tris–HCl pH8, 0.05% Tween 20, 0.1% sodium dodecylsulphate (SDS), 14 mM β-mercaptoethanol, 200 mM dithiothreitol, 200 mM sucrose, Roche complete mini EDTA-free protease inhibitor cocktail] was added to the ground leaf material. Ground samples were placed on ice and mixed for 2 min using a mechanical pestle and centrifuged at 700 *g* for 15 min at 4 °C to obtain the supernatant (soluble fraction). The pellet (insoluble fraction) was resuspended with equal volume of the protein extraction buffer and sonicated for 30 s. The supernatant and pellet were subjected to Bradford analysis to determine the total protein concentration.

After estimation of TSP using Bradford method, 2/3 μg of TSP from sample was separated in 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes for immunoblotting as described earlier (Verma *et al.*, 2008). The protein separated

by SDS-PAGE gel was transferred to nitrocellulose membrane by electroblotting and the membrane was blocked overnight with 3% non-fat dry milk. To detect CTB-AMA1 and CTB-MSP1-fused proteins, blots were incubated with 1 : 3000 rabbit anti-CTB primary polyclonal antibody (Sigma, St Louis, MO, USA) followed by 1 : 5000 HRP-conjugated donkey anti-rabbit secondary antibody (Southernbiotech, Birmingham, AL, USA). A SuperSignal® West Pico chemiluminescence substrate Kit (Pierce, Rockford, IL, USA) was used for autoradiographic detection.

For enrichment, chloroplast-derived CTB-malarial proteins were extracted from 10 g freeze-dried leaf materials. The samples were placed on ice and homogenized for 5 min with an OMNI International (GLH-2596) probe and centrifuged at 700 g for 15 min at 4 °C. The supernatant was collected and then subjected to TALON superflow Metal Affinity Resin (Clontech) to enrich the chloroplast-derived CTB-malarial proteins according to manufacturer's instructions. The eluted fraction was collected and subjected to the Bradford Protein assay (BioRad) and to the RC-DC Protein Assay (Bio-Rad). The eluted fractions were dialysed with sterile phosphate-buffered saline (PBS) and the Slide-A-Lyzer Dialysis Cassette 10 000 MW (Pierce).

Cholera toxin-B-GM1-ganglioside receptor binding assay

A 96 well microtiter plate was coated with GM1-ganglioside receptor (Sigma G-7641) by incubating 100 μL GM1 (3 μg/mL) in bicarbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) at 4 °C overnight. The wells were blocked with 200 μL of 3% fat-free milk in PBST (PBS with 0.05% Tween-20) for 2 h followed by three washes with PBST and water. The plate was incubated with various concentrations of TSP from leaves of transformed and untransformed plants in bicarbonate buffer (100 μL per well) overnight at 4 °C. The plate was washed three times with PBST and water, the wells were blocked with 200 μL per well of 3% fat-free milk in PBST at 37 °C for 2 h followed by three washes with PBST and water. The plate was incubated with 1 : 4000 dilution of rabbit anti-CTB antibody for 2 h followed by three washes with PBST and water, followed by incubation for 2 h with 1 : 4000 dilution of goat-anti-rabbit IgG, conjugated to horseradish peroxidase. Colour was developed using tetra methyl benzidine (TMB) as substrate. Reaction was stopped by adding 50 μL of 2 N H_2SO_4 and the absorbance was read on a plate reader (BioRad) at 450 nm.

Enzyme-linked-immunosorbent serologic assay

Sandwich/antigen-specific ELISA was performed as described earlier (Arlen *et al.*, 2008). In malaria studies, we used sera from bleed number 4 and 5 at dilution cited in the text, however, in cholera studies sera from terminal bleeding was used except stated otherwise at dilution mentioned in the text.

Mice and immunization schedule

Enriched chloroplast-derived proteins (~2.5 mg) from transgenic tobacco crude extract was mixed with 1 : 4 diluted Alhydrogel (Aluminium Hydroxide Gel; Sigma) in PBS and incubated overnight with gentle rocking at 4 °C. The samples were centrifuged at 2000 g for 5 min at 4 °C. The RC-DC Protein Assay (BioRad) was used to determine the adsorption efficiency by comparing the total amount of protein added with the adjuvant and the protein remaining in the supernatant after binding to adjuvant. The protein-adsorbed pellet was resuspended in sterile PBS to a final concentration of 1 μg/μL. For subcutaneous injections, 100 μL of Alhydrogel or chloroplast-derived protein (25 μg) adsorbed to Alhydrogel was injected into the scruff of the neck using a tuberculin syringe fitted with a 27-G needle. The leaf material for oral delivery was ground in liquid nitrogen with mortars and pestles and stored at −80 °C until the day of immunization. Oral doses (500 mg each) of either untransformed or transgenic leaf material was resuspended in 200 μL of sterile PBS and homogenized on ice for 5 min with an OMNI International (GLH-2596) probe. The plant cell suspension was stored on ice until

oral delivery. The plant cell suspension (200 μL) was delivered via oral gavage by using a tuberculin syringe and a 20-G bulb-tipped gastric gavage needle.

Ninety female BALB/c mice, purchased from the Charles River Laboratories at 7 week of age, were housed at the University of Central Florida mouse facility in ventilated cages under specific pathogen-free conditions. All mice and procedures performed in this study are based on an approved protocol and are in accordance with the UCF-IACUC. Mice were randomly divided into nine groups (n = 10 per group): group 1: oral UT group gavaged with untransformed leaves; group 2: adjuvant with no bound antigen; group 3: CTB-AMA1 purified antigen with adjuvant; group 4: oral gavage with leaves expressing CTB-AMA1; group 5: CTB-MSP1 purified antigen with adjuvant; group 6: oral gavage with leaves expressing CTB-MSP1; group 7, CTB-MSP1 and CTB-AMA1 purified antigens bound with adjuvant; group 8: oral gavage with leaves expressing CTB-AMA1 and MSP1 and group 9: untreated mice. Mice in groups 2–8 were initially primed subcutaneously with corresponding antigen followed by oral and/or subcutaneous boosts in the course of this study. Mice in subcutaneous group received boosts on days 13, 27, 43, 55, 155 and 219. Mice in oral gavage group received boosts on days 10, 17, 24, 31, 37, 45, 52, 59, 150, 157, 189 and 219. Final oral and subcutaneous boosts were given on day 219 and mice were challenged with CT toxin 2 weeks after the final boost.

Cholera toxin challenge and intestinal water content

Cholera toxin (CT) (C8052; Sigma) was diluted (final concentration 1 mg/mL) in PBS buffer containing 6% $NaHCO_3$ and 0.5% albumin. Effective dose of CT was determined empirically and unvaccinated BALB/c mice with the same age and sex as of our experimental group were given different doses of CT (1, 1.5, 2, 3 and 4 μg/g of body weight) for 14 h. Based on these data, mice were challenged with a CT dose of 1.5 μg/g of body weight. The mice remained in their cages without food but water *ad libitum*. The mice were scarified after 14 h and intestinal water retention was collected and measured.

Measurement of immunoglobulin and cytokines

All sera used in this study for cytokines and antibodies detection was from terminal bleeding except stated otherwise. Sandwich ELISA was performed on transgenic lettuce leaf materials expressing CTB and mice sera for cytokines detection (Arlen *et al.*, 2008). Sandwich ELISA for different cytokines was performed as described (Arlen *et al.*, 2008). Plates (96-well) were coated with anti-mouse IL-2 (2 μg/mL), IL-4 (2 μg/mL), IFNγ (2 μg/mL), IL-17A (2 μg/mL) antibodies (all from eBiosciences) using carbonate buffer (pH = 9.6) at 4 °C for 12–16 h. Plates were washed and hybridized with diluted sera at 37 °C for 1 h. Detection was performed as described earlier. Capture ELISA for CTB-antigen-specific IgA, IgG1, IgG2a, IgG2b, IgG3, IgM antibodies in sera and intestinal content (IgA only) of different group of mice were performed by coating 96-well flat bottom plate with 1 μg/mL (100 μL) of CTB (Sigma) in carbonate buffer (pH = 9.7) at 4 °C for 12–16 h. All rat anti-mouse IgG1 antibodies were purchased from Southern Biotech, AL.HRP-conjugated streptavidin (1 : 4000; Peirce) and TMB were used for detection and substrate respectively. For CTB-antigen-specific IgA in sera and intestinal content, goat-anti-mouse IgA-HRP (1 : 2000l; American Qualex) was used. Rabbit anti-CTB Ab (1 : 4000; Sigma) and anti-rabbit IgG-HRP Ab (1 : 7500) were used as primary and secondary antibodies for CTB respectively.

Flow cytometry analysis

Flow cytometry analysis was performed on fresh single-cell suspension of splenocytes. Cell surface staining on freshly prepared splenocytes was performed using anti-mouse CD4 (L3T4; BD Pharmingen), CD25 (3C7; BD Pharmingen), CD127 (IL-7Rα) (SB/199; BD Pharmingen), CD44 (IM7; BD Pharmingen), CD11c (HL3; BD Pharmingen), CD80 (16-10A1; eBioscience),

biotin-conjugated MHC II (M5/114.15.2; eBioscience). Purified rat anti-mouse CD16/CD32 (2.4G2; BD Pharmingen) was used to block Fc receptor in myeloid cell lineages.

Intra-cellular staining of Foxp3 (FJK, 16S; eBioscience), IL-4 (11B11; eBiosciences), IL-10 (JES5-16E3; eBioscience), IFNγ (XMG1.2; eBioscience) was performed using foxp3 intra-cellular staining kit (eBioscience) according to instructions provided by manufacturer. Flow cytometry was performed using FACSCalibur (BD Bioscience) and 30 000 events were acquired for each condition and data analysis was performed using FCS express (v3) software (De Novo soft ware).

Immunofluorescence detection of malarial antigens with sera of vaccinated mice

A revised protocol was adopted for the preparation and fixation of RBC (Tonkin *et al.*, 2004) and for detection of antigen with immunofluorescence (Ayong *et al.*, 2007). Diluted sera (1 : 500) were hybridized on RBC followed by hybridization with diluted (1 : 1000) Alexa Fluor 555 goat-anti-mouse antibody. Cells were allowed to settle on previously coated coverslips with 1% PEI for 30 min at room temperature. The mounting solution, 50% glycerol with 0.1 mg/mL of DABCO (Sigma) was added to cover slips and then inverted on microscope slides. Fluorescence images were observed and captured by the LSM 510 confocal laser scanning microscope (Carl Zeiss).

In vitro parasite inhibition assay

The 3D7 *P. falciparum* culture was synchronized with ring stage parasites with sorbitol lysis. The parasite completed one cycle and was allowed to mature to the trophozoite-schizont stage. The haematocrit and parasitemia were adjusted to 2% (2.5% parasitemia for the MRA-35 PfMSP1$_{19}$ *in vitro* parasite inhibition assay). Mouse sera (bleed 4) and MRA-35 PfMSP1$_{19}$ (positive control) were heat inactivated at 56 °C for 30 min and hybridized on human RBC overnight at 4 °C (Sachdeva *et al.*, 2006). The mouse serum was added to the parasite culture in 96-well plates at a final concentration of 20% (for the MRA-35 PfMSP1$_{19}$ *in vitro* parasite inhibition assay 5 μL of antibody was added and diluted 1 : 5–1 : 625 to 25 μL of parasite culture). To serve as a negative control, no serum was added to wells and replaced with culture media. The cultures were incubated for 48 h to allow for schizont rupture and merozoite invasion. The assays were preformed in duplicate and repeated at least three times.

For microscopic analysis using the 100× oil immersion lens, blood smears were made and stained with Giemsa and the numbers of parasites per 900–1100 RBC were determined for each well. Parasitemia was measured using the following formula (infected RBC/infected + uninfected RBC) ×100. Per cent of inhibition was determined by the following formula (% parasitemia of no sera added − % parasitemia of experimental mouse sera/% parasitemia of no sera added) ×100. Relative per cent of inhibition was determined by the following formula [% of inhibition from experimental mouse sera/% inhibition of MRA-35 PfMSP1$_{19}$ (positive control)] ×100 and the per cent of inhibition for the positive control was set at 100%.

Statistical analysis

Data are reported as the mean ± SD. All analyses for statistically significant differences were performed using one-way ANOVA and the *t*-test (GraphPad Prism 5) and *P* values <0.05% considered significant.

Acknowledgments

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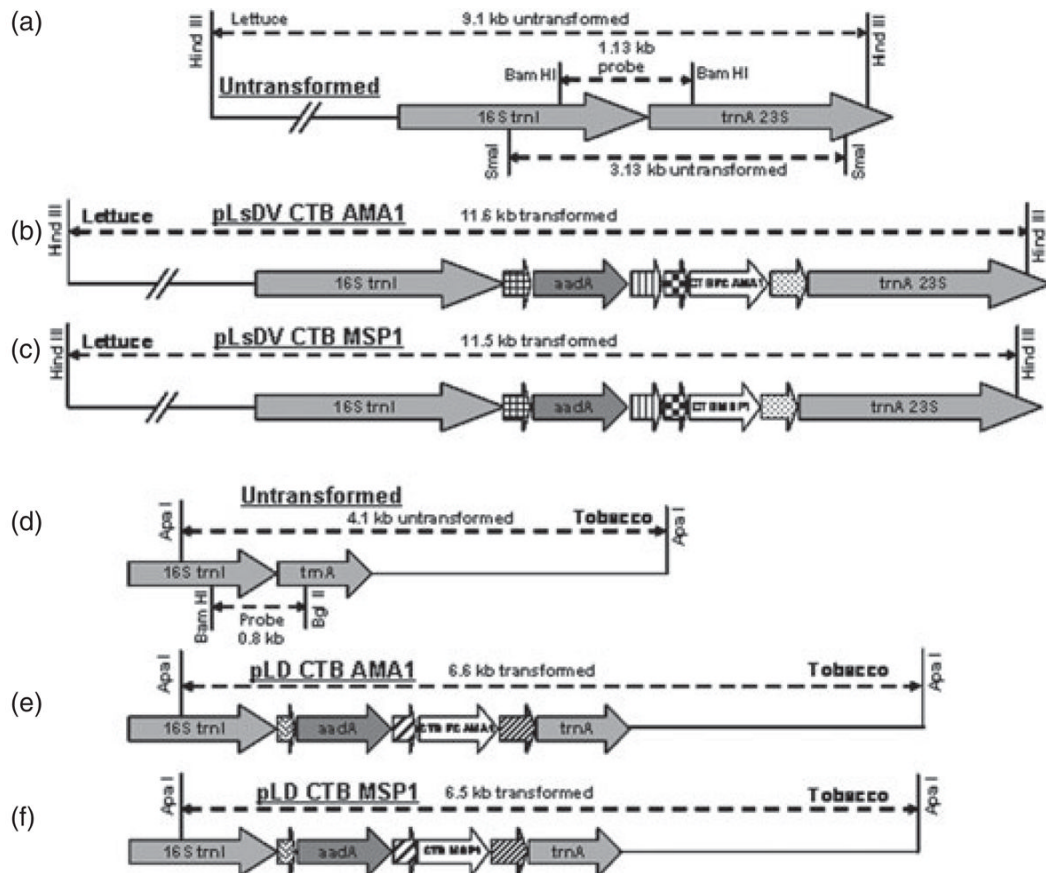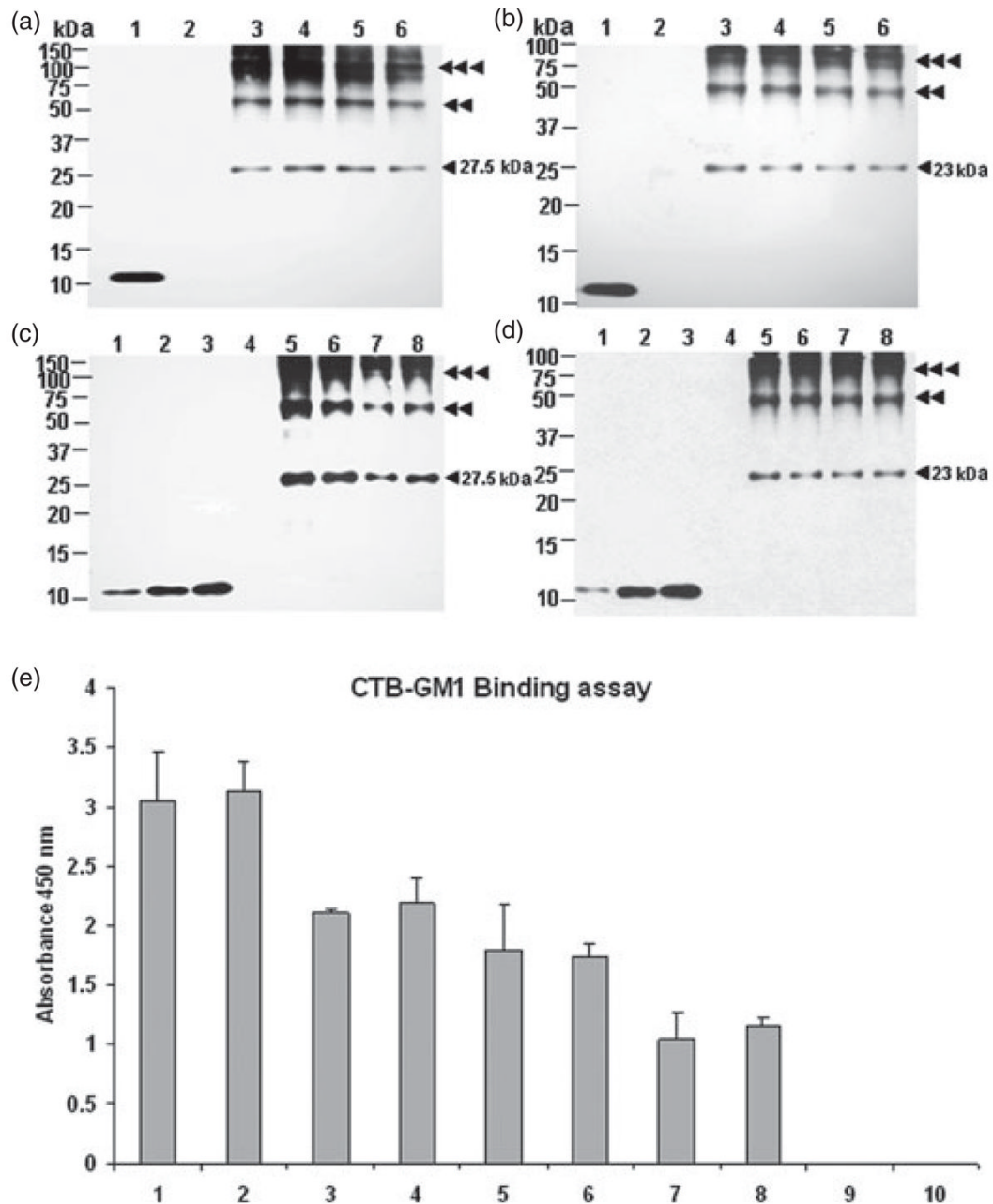
Figure 1.
Schematic representation of the lettuce and tobacco chloroplast constructs. Schematic representation of the lettuce and tobacco chloroplast genome-flanking sequences used for homologous recombination, probe DNA sequence and chloroplast transformation vectors including the transgene cassettes for cholera toxin-B subunit (CTB)-apical membrane antigen-1, CTB-merozoite surface protein-1, integration sites and anticipated products of the transplastomic lines in Southern blots. ◄ represents lettuce 16s ribosomal operon promoter; ◄ represents lettuce 3′ *rbc*L; ◄ represents lettuce *psb*A promoter including 5′-untranslated region (UTR); ◄ represents lettuce *psb*A 3′UTR; ◄ represents tobacco *psb*A promoter including 5′UTR; ◄ represents tobacco *psb*A 3′UTR; ◄ represents tobacco 16s ribosomal operon promoter.
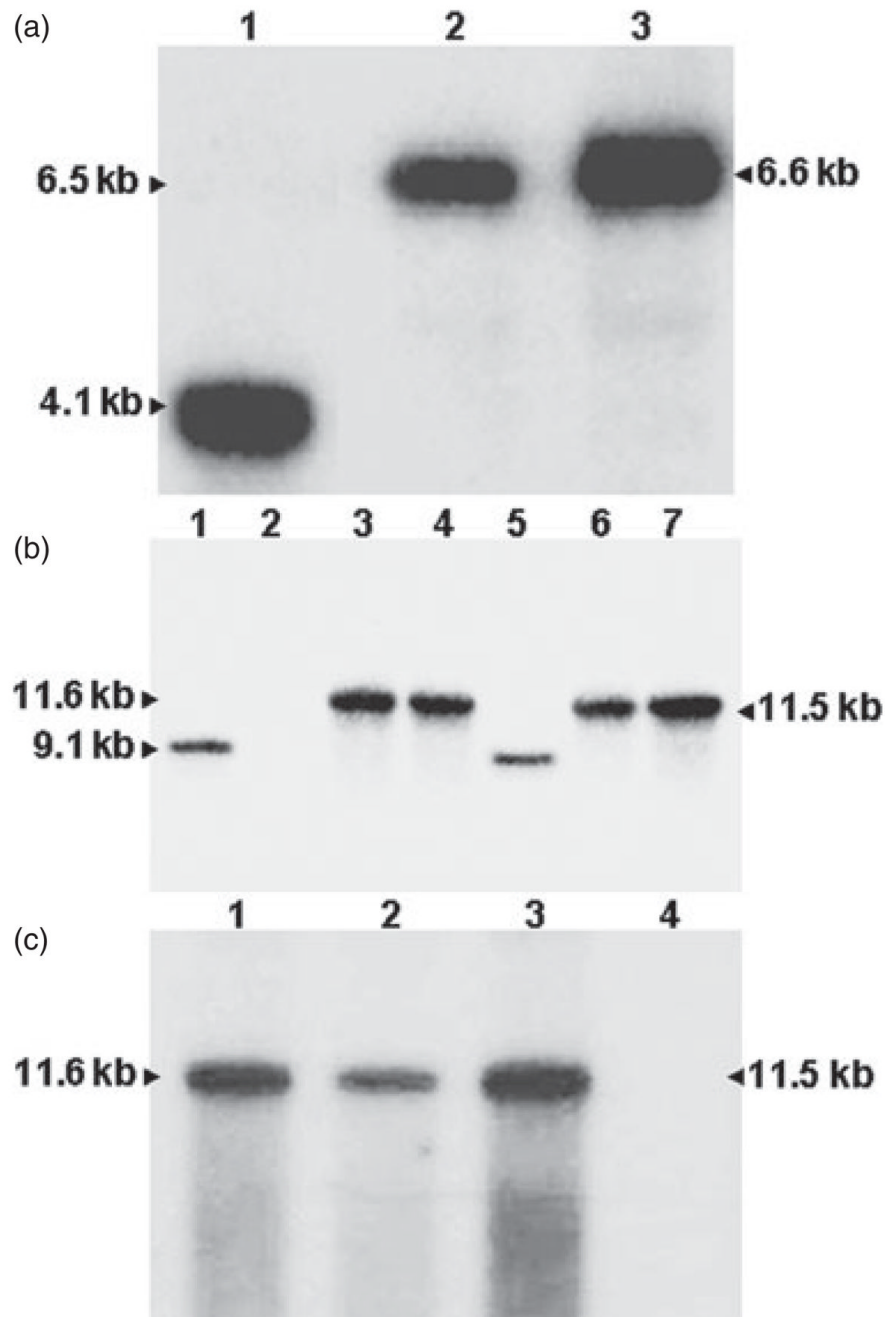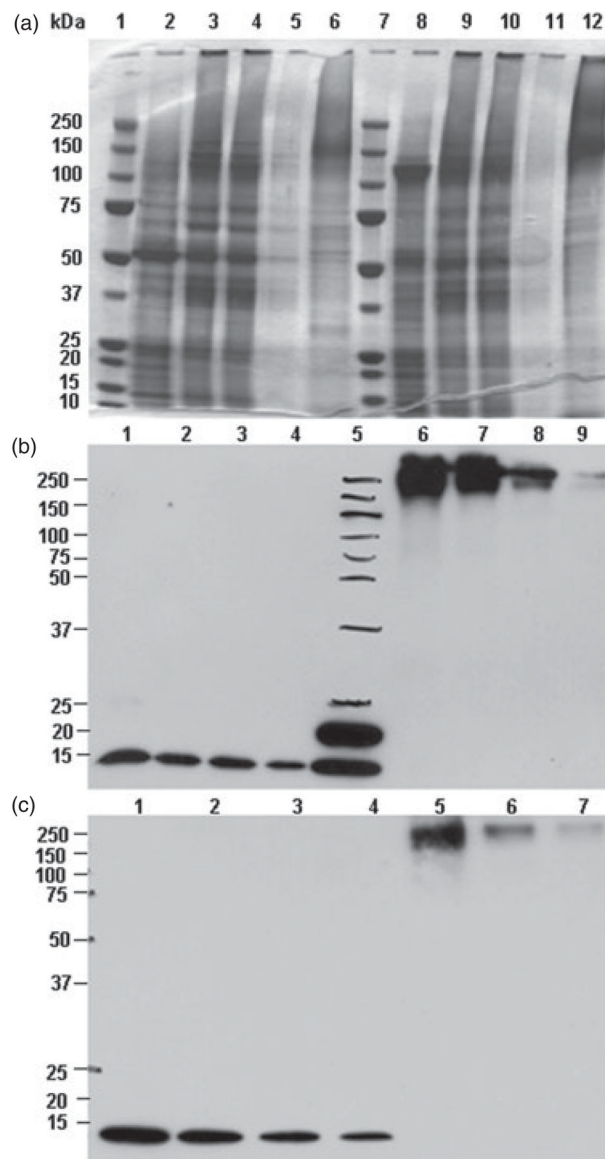
Figure 2.
Southern blots analyses of transgenic plants. Genomic DNA (2 μg) was digested with *Hin*dIII in lettuce and *Apa*I in tobacco untransformed and transplastomic lines. The blots were hybridized with the lettuce or tobacco-flanking sequence or cholera toxin-B subunit (CTB) probes. (a) Tobacco; lane 1: untransformed (4.1 kb), lane 2: homoplasmic CTB-merozoite surface protein-1 (MSP1; 6.5 kb) and lane 3: homoplasmic CTB-apical membrane antigen-1 (AMA1) (6.6 kb) lines. (b) Lettuce; lane 1: untransformed (9.1 kb), lane 2: blank, lanes 3 and 4: homoplasmic CTB-AMA1 (11.6 kb) lines, lane 5: untransformed, lanes 6 and 7: homoplasmic CTB-MSP1 (11.5 kb) lines. (c) Lettuce probed with CTB. Lanes 1–3: transplastomic lines, lane 4: untransformed.

Figure 3.
Expression of vaccine antigens in transgenic chloroplasts. Tobacco (2 μg) or lettuce (3 μg) of TSP were separated on SDS-PAGE, transferred on nitrocellulose membrane and hybridized with the cholera toxin-B subunit (CTB) primary antibody. (a) CTB-apical membrane antigen-1 (AMA1) in tobacco and lettuce. Lane 1: standard CTB (100 ng), lane 2: untransformed extract, lanes 3 and 4: Tobacco CTB-AMA1 supernatant and pellet, lanes 5 and 6: Lettuce CTB-AMA1 supernatant and pellet. (b) CTB-merozoite surface protein-1 (MSP1) in tobacco and lettuce. Lane 1: CTB standard (100 ng), lane: 2 untransformed extract, lanes 3 and 4: tobacco CTB-MSP1 supernatant and pellet, lanes 5 and 6: lettuce CTB-MSP1 supernatant and pellet. (c) CTB-AMA1 in tobacco and lettuce with known quantities of the CTB standard. Lanes 1–3: CTB standard (50, 100 and 200 ng), lane 4: untransformed extract, lanes 5 and 6: tobacco CTB-

AMA1 T0 and T1 transplastomic lines, lanes 7 and 8: lettuce CTB-AMA1 T0 and T1 transplastomic lines. (d) CTB-MSP1 in tobacco and lettuce with known quantities of CTB standard. Lanes 1–3: CTB standard (50, 100 and 200 ng), lane 4: untransformed extract, lanes 5 and 6: tobacco CTB-MSP1 T0 and T1 transplastomic lines, lanes 7 and 8: lettuce CTB-MSP1 T0 and T1 transplastomic lines. Monomers, dimers and pentamers are shown with arrows. (e) CTB-GM1 binding assay of CTB-AMA1 and CTB-MSP1 tobacco and lettuce T0 and T1 transplastomic lines. Lanes 1 and 2: tobacco CTB-AMA1 T0 and T1, lanes 3 and 4: lettuce CTB-AMA1 T0 and T1, lanes 5 and 6: tobacco CTB-MSP1 T0 and T1, lanes 7 and 8: lettuce CTB-MSP1 T0 and T1 transplastomic lines, lane 9: untransformed plant, lane 10: phosphate-buffered saline.

Figure 4.
Enrichment of chloroplast-derived cholera toxin-B subunit (CTB) malarial antigens. (a) CTB
FC apical membrane antigen-1 (AMA1) protein was extracted from transformed leaves and
the crude extract was subjected to Talon Superflow Metal Affinity Resin and analysed.
Molecular size standards are indicated in lanes 1 and 7. Lanes 2–6: reduced and lanes 8–12:
non-reduced conditions of CTB FC AMA1 protein enrichment was observed by using a
gradient gel (4%–12%) and gel electrophoresis. The following proteins are visualized: lanes 2
and 8: untransformed, lanes 3 and 9: lysate, lanes 4 and 10: flow through, lanes 5 and 11: wash,
and lanes 6 and 12: enriched protein. (b) Immunoblot analysis of tobacco CTB FC AMA1
using CTB primary antibody, lanes 1–4: CTB standard (1000, 500, 250 and 125 ng
respectively), lane 5: protein marker, lanes 6–9: eluted CTB FC AMA1 (1.5, 0.75, 0.375 and
0.1875 μg respectively). (c) Immunoblot analysis of tobacco CTB-merozoite surface protein-1
(MSP1) using CTB primary antibody. Lanes 1–4: CTB standard (1000, 500, 250 and 125 ng
respectively), lanes 5–7: eluted CTB MSP1 (1.5, 0.75 and 0.375 μg respectively). Eluted

proteins and CTB were subjected to densitometry to determine the enrichment of CTB FC AMA1 and CTB-MSP1 to be administered to mice for subcutaneous injection.
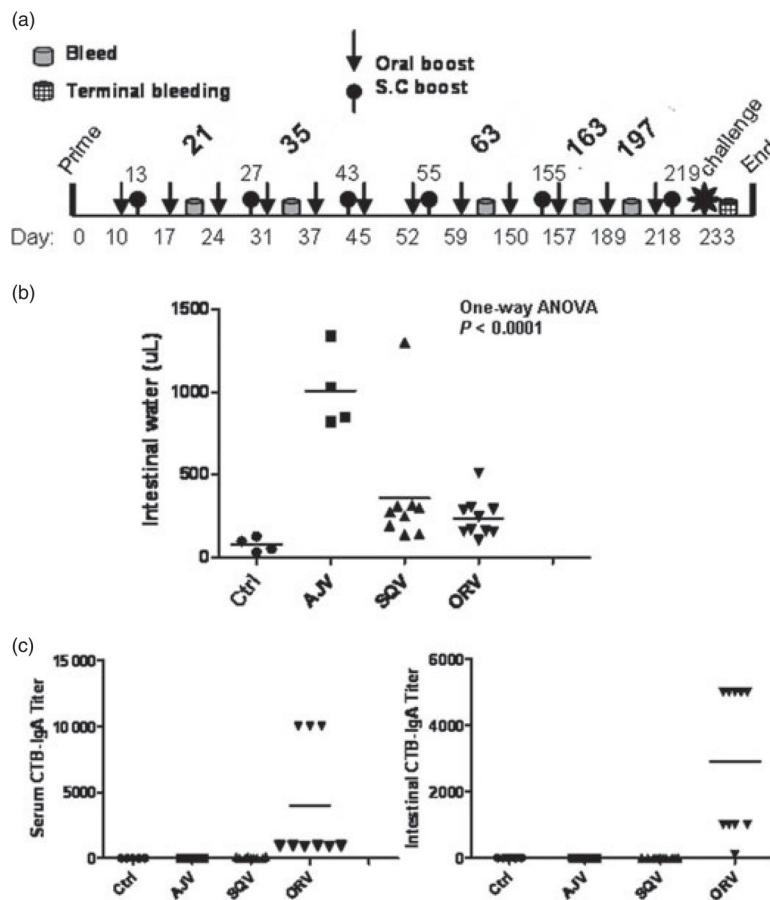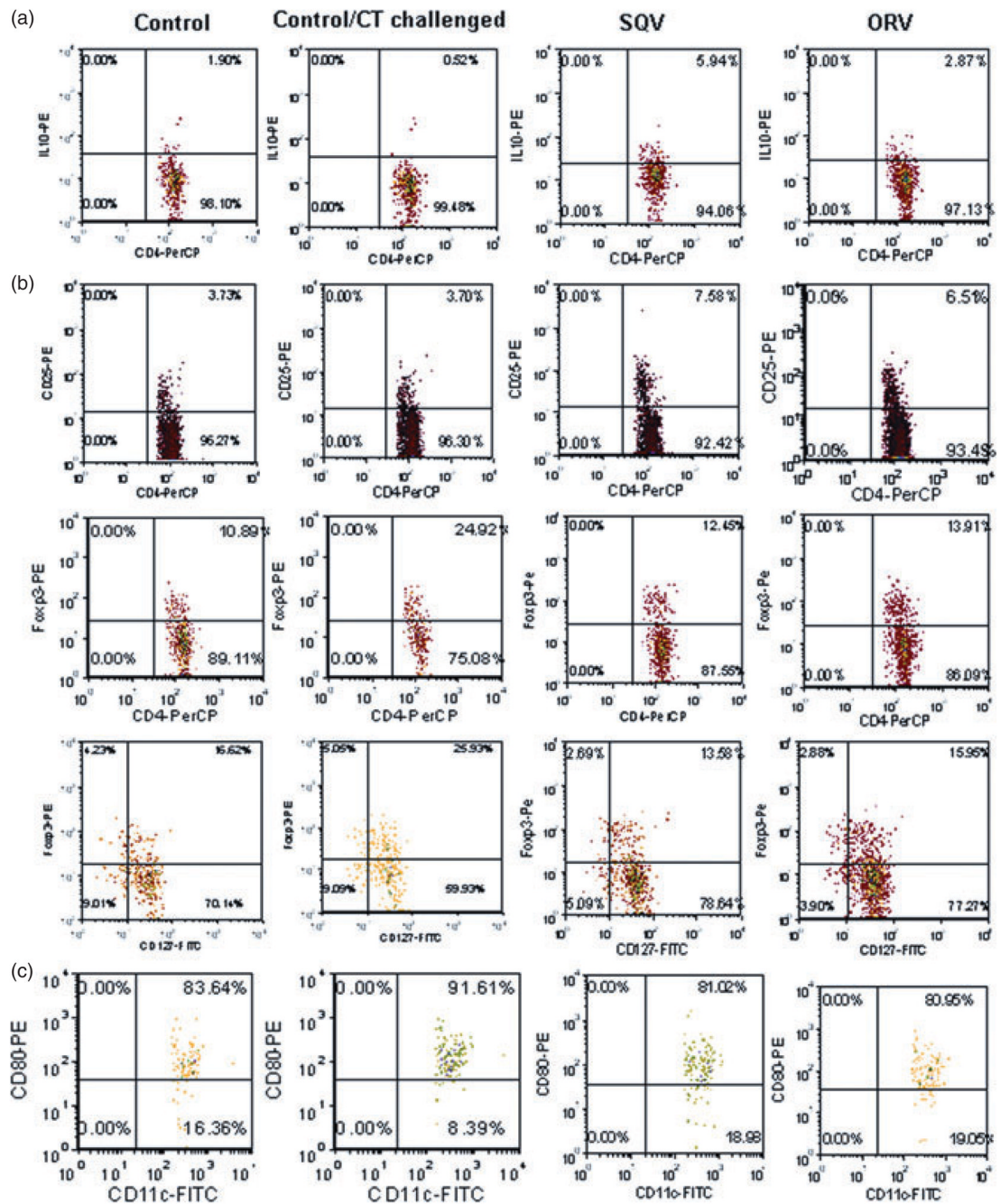
Figure 5.
The experimental design and evaluation of cholera toxin (CT) challenge on control and immunized mice. (a) The immunization schedule is shown by a horizontal line. Arrows from left to right show initial priming on day 0. Mice received twelve oral boosters (small vertical arrows) and six subcutaneous boosters (short bars with solid circles). Mice were bled five-times (grey-filled cylinder) and terminal bleeding (hatched cylinder) was performed 14 h post-challenge. Days of oral and s.c boosts are shown below and on the top of the horizontal arrow respectively. Days of bleeding are shown on top of each cylinder. (b) CT challenge in control and vaccinated mice. CT (1.5 μg/g of body weight) was challenged orally for 14 h. Each spot represents intestinal water content (μL) of an individual mouse in different groups after CT challenge (one-way ANOVA, $P < 0.0001$). Each spot represents intestinal water content (μL) of an individual mouse in different groups after CT challenge (one-way ANOVA, $P < 0.0001$). (c) CTB-antigen-specific serum and intestinal IgA in different groups of mice measured by antigen-specific enzyme-linked-immunosorbent serologic assay. Control (Ctrl), adjuvant (ADJ), subcutaneous vaccinated (SQV) and orally vaccinated (ORV) mice.
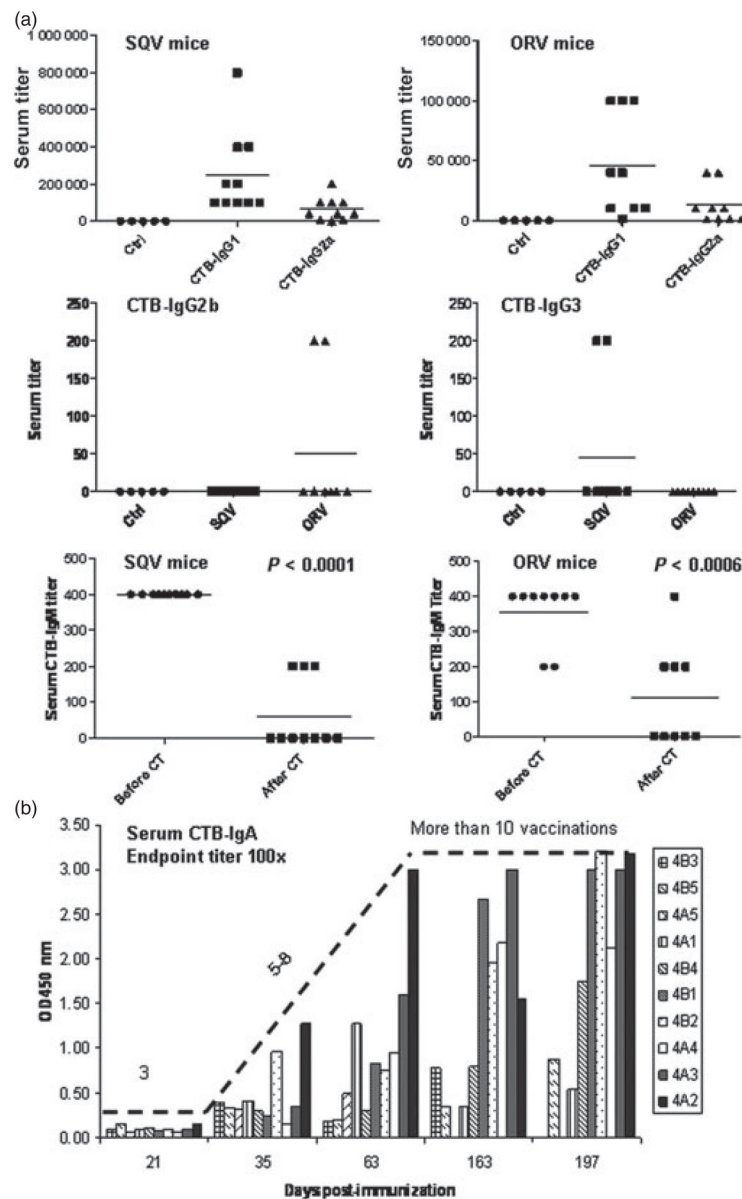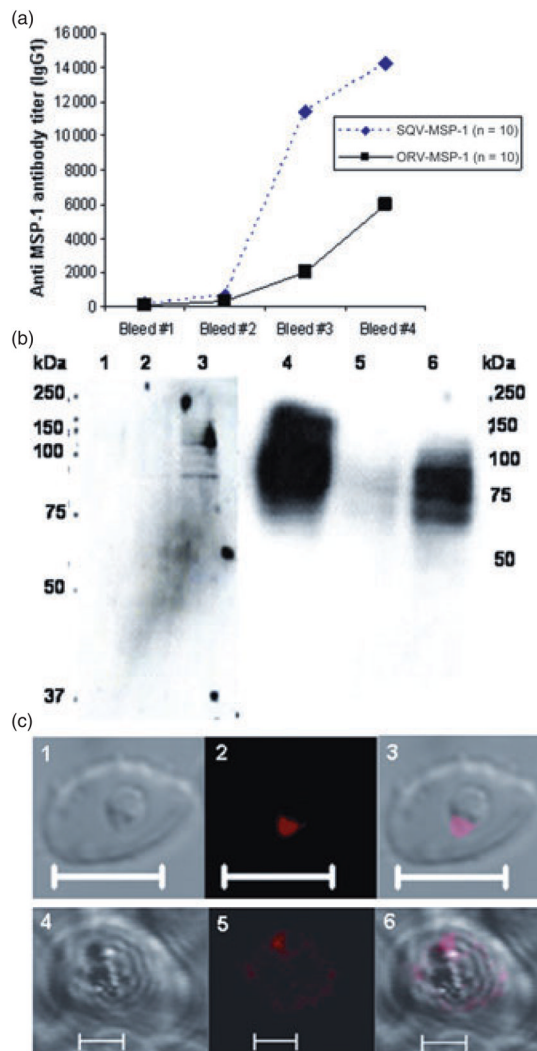
Figure 6.
Mechanisms of generated immunity in vaccinated and control mice. (a) Sera of SQV and ORV-cholera toxin-B subunit (CTB) mice were evaluated for antigen-specific immunoglobulins using CTB-specific enzyme-linked-immunosorbent serologic assay as shown in each panel. Top row shows CTB-IgG1 and -IgG2a titres; middle row shows CTB-IgG2b and IgG3 titres; the bottom row shows serum CTB-IgM titres before and after CT challenge in oral and subcutaneously immunized mice. Data represent one of at least 3–5 independent experiments for any given immunoglobulin. Ctrl, control; SQV, subcutaneous immunization; ORV, oral immunization. (b) Determination of effectiveness of numbers of boosters to generate CTB-specific serum IgA in orally gavaged mice with transplastomic leaves. Ten-week-old mice were boosted subcutaneously (until 189 days) or orally (until 219 days). Sera were collected until 197 days post-immunization.

Figure 7.
Single-cell analyses of pooled splenocytes from vaccinated and control mice. (a, b) Flow cytometry analyses were performed on fresh single-cell suspension of pooled splenocytes obtained from unimmunized/control, unimmunized/cholera toxin (CT) challenged, subcutaneous SQV and orally ORV-CTB immunized mice after CT challenge as described in Experimental procedures. Shown in rows 1–4 is gated splenocytes CD4+ T cells population and cells were stained with anti-CD4 antibody followed by staining with anti-interleukin-10 (IL10), CD25, Foxp3 and CD127 antibodies respectively. Intracellular staining of Foxp3 and IL-10 was performed using Foxp3 intracellular staining kit (eBioscience) according to instructions provided by manufacturer. Shown is gated CD4+ T cells population. (c) Post-

challenge analysis. Pooled splenocytes of different groups as depicted were stained with cell surface markers and staining was performed using anti-mouse CD11c-FITC, CD80-PE and biotin-conjugated MHC II and then stained with streptavidin-conjugated PerCP (BD Bioscience). Shown is gated splenocytes CD11C+ gated population (bottom row) and cells were further stained with anti-CD80 antibody. Purified rat anti-mouse CD16/CD32 was used for 10 min to block Fc receptor before initiation of cell surface staining. Shown are gated on CD11c^{+high} splenic dendritic cells.

Figure 8.
Cross-reactivity of antisera generated against transplastomic malarial vaccine antigens. (a) Detection of anti-merozoite surface protein-1 (MSP1)$_{19}$ IgG1 antibody in sera of mice immunized by subcutaneous (SQV) and oral (ORV)-MSP1 delivery. Sera of SQV and ORV-MSP1 mice were subjected to an antigen-specific MSP1-IgG1 enzyme-linked-immunosorbent serologic assay. (b) Immunoblot analysis: 36.8 μg of cell-free parasite extracts from ring, trophozoite and schizont stages were resolved on sodium dodecylsulphate polyacrylamide gel electrophoresis gels and were subjected to immunoblot analysis using diluted sera from immunized mice. Sera collected from immunized mice (bleed 4 and was diluted 1 : 5) recognized the native 83-kDa apical membrane antigen-1 (AMA1) protein (lanes 1–3) and the native 190 kDa MSP-1 protein (lanes 4–6). The parasite stages analysed from *Plasmodium falciparum* 3D7 culture were ring: lanes 1 and 4, trophozoite: lanes 2 and 5 and schizont: lanes 3 and 6. (c) Immunofluorescence analysis: *P. falciparum* 3D7 parasites were immunostained with anti-AMA1 (top row) and anti-MSP1 antibodies (lower row) from immunized mice. Panels 1 and 4 are differential interference contrast images, panels 2 and 5 are fluorescence images, and panel 3 and 6 are merged images of previous two panels. The AMA1 antibodies recognized the apical end of the parasite in the ring developmental stage of intraerythrocytic growth (1, 2 and 3). The MSP-1 sera from immunized mice (bottom row) detected the

developing merozoites at the schizont stage of the parasitic growth (4, 5 and 6). Bar size = 10 μm.

Table 1

Parasitemia assays and relative inhibition of parasite in red blood cells (RBC) using sera of immunized and control mice

Group	Parasitemia (%)	Mean parasitemia (%)	Relative inhibition (%)
No Ab	6.6–6.7	6.6	–
MRA-35 PfMSP1–19	2.5–3.5	3.1	100.0
Group 1 (oral WT)	5.9–6.6	6.1	14.3
Group 2 (s.c. adjuvant)	5.5–6.2	5.8	22.8
Group 3 (s.c. AMA1)	2.8–3	2.9	105.8
Group 4 (oral AMA1)	2.4–3.3	3.0	102.8
Group 5 (s.c. MSP1)	2.4–2.6	2.5	117.2
Group 6 (oral MSP1)	2.6–3.6	3.2	97.2
Group 7 (s.c. AMA1 and MSP1)	2.7–3.9	3.3	94.3
Group 8 (oral AMA1 and MSP1)	3.3–3.8	3.6	85.8
Group 9 (untouched)	5.6–5.8	5.7	25.7

Average parasitemia and relative inhibition was determined as shown. The stage of parasite used was trophozoite-schizont and the haematocrit and parasitemia were adjusted to 2%. Control and experimental mouse sera were heat inactivated and incubated with uninfected RBC overnight at 4 °C. The mouse serum was added to the parasite culture at a final concentration of 20%. The cultures were incubated for 48 h to allow for schizont rupture and merozoite invasion. Assays were preformed in duplicate and repeated at least three times. Parasitemia was determined and the relative per cent of inhibition was calculated by using the formula described in Experimental procedures.

AMA1, apical membrane antigen-1; merozoite surface protein-1.