Phase I Trial of an Alhydrogel Adjuvanted Hepatitis B Core Virus-Like Particle Containing Epitopes of Plasmodium falciparum Circumsporozoite Protein

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Abstract

The objectives of this non-randomized, non-blinded, dose-escalating Phase I clinical trial were to assess the safety, reactogenicity and immunogenicity of ICC-1132 formulated with Alhydrogel (aluminum hydroxide) in 51 healthy, malaria-naive adults aged 18 to 45 years. ICC-1132 (Malarivax) is a recombinant, virus-like particle malaria vaccine comprised of hepatitis core antigen engineered to express the central repeat regions from Plasmodium falciparum circumsporozoite protein containing an immunodominant B [(NANP)3] epitope, an HLA-restricted CD4 (NANPNVDPNANP) epitope and a universal T cell epitope (T*) (amino acids 326—345, NF54 isolate). We assessed an Alhydrogel (aluminum hydroxide)-adjuvanted vaccine formulation at three ICC-1132 dose levels, each injected intramuscularly (1.0 mL) on study days 0, 56 and 168. A saline vaccine formulation was found to be unstable after prolonged storage and this formulation was subsequently removed from the study. Thirty-two volunteers were followed for one year. Local and systemic adverse clinical events were measured and immune responses to P. falciparum NF54 were determined utilizing the following assays: IgG and IgM ELISA, indirect immunofluorescence against P. falciparum sporozoites, circumsporozoite precipitin (CSP) and transgenic sporozoite neutralization assays. Cellular responses were measured by proliferation and IL-2 assays. Local and systemic reactions were similarly mild and well tolerated between dose cohorts. Depending on the ICC-1132 vaccine concentration, 95 to 100% of volunteers developed antibody responses to the ICC-1132 immunogen and HBc after two injections; however, only 29—75% and 29—63% of volunteers, respectively, developed malaria-specific responses measured by the malaria repeat synthetic peptide ELISA and IFA; 2 of 8 volunteers had positive reactions in the CSP assay. Maximal transgenic sporozoite neutralization assay inhibition was 54%. Forty-seven to seventy-five percent demonstrated T cell proliferation in response to ICC-1132 or to recombinant circumsporozoite protein (rCS) NF-54 isolate. This candidate malaria vaccine was well tolerated, but the vaccine formulation was poorly immunogenic. The vaccine may benefit from a more powerful adjuvant to improve immunogenicity.

Trial Registration: ClinicalTrials.gov NCT00587249

Introduction

An effective vaccine is needed to prevent or attenuate disease from Plasmodium falciparum malaria, the most important cause of malaria morbidity and mortality throughout the world [1]. Protection from P. falciparum malaria infection and challenge was first demonstrated following immunization of humans with irradiation-attenuated P. falciparum sporozoites [2]. High levels of antibody directed against repeat regions of the circumsporozoite protein (CS) and high levels of interferon (IFN)-γ production by CD4+ and CD8+ cells against epitopes of CS, is associated with protection of humans and primates from P. falciparum malaria [3–7]. These findings suggest that a subunit vaccine which elicits robust humoral immunity directed against the extracellular sporozoite and robust cellular immunity with which to eliminate infected hepatocytes, could prevent patent blood-stage infection, the stage of the infection responsible for clinical illness.

Virus-like particles have been used recently as highly immunogenic delivery platforms for a variety of vaccines [8–13]. The virus-like particle malaria vaccine RTS,S is composed of hepatitis B virus
surface antigen which contains the CS repeat and C terminus region (amino acids 207—395) of the \textit{P. falciparum} NF54 isolate (3D7 clone). In combination with potent proprietary adjuvants, this vaccine has protective efficacy against malarial disease and severe malaria [14–17]. Complete protection was obtained in 40% of immunized malaria naive volunteers undergoing sporozoite challenge [15,18] and 30% protection against the first clinical episode of malaria for 18 months in children living in malaria endemic areas [16,17]. More robust vaccines capable of greater and longer duration of protection are sought through more effective vaccine delivery platforms. The hepatitis B virus core protein (HBc) has been demonstrated to be an effective malaria vaccine platform in animals where high levels of anti-CS repeat antibodies protected animals from malaria challenge [19,20].

Circumsporozoite protein is comprised of a central portion of amino acid repeats (NANP) representing dominant T cell-dependent B cell epitopes [21,22]. T cell epitopes have been identified in the CS molecule, which are HLA-restricted CD8$^+$ and CD4$^+$ T cell epitopes, as well as universal CD4$^+$ T cell epitopes [6,23–25]. The present vaccine, ICC-1132, was conceived in an effort to boost antibody levels and generate a robust cellular immune response. It is comprised of central repeat regions of CS containing (1) both immunodominant B (NANP)$_3$ and HLA-restricted CD8$^+$ T cell (NANPNVDP) epitopes identified from irradiated sporozoite immunization studies [5,21], (2) a universal T cell epitope (T$^*$) from the carboxyl terminus of CS (amino acids 326—345 NF54 isolate) containing CD4$^+$ T cell epitopes, which bind to a wide range of HLA types [24,26,27], and (3) at least one CD8$^+$ T cell epitope [28]. These CS epitopes are inserted into a HBc backbone which spontaneously aggregates to form virus-like particles. This vaccine was found to be highly immunogenic in rodent and non-human primates [29].

After initiation of this present study in the USA, the ICC-1132 vaccine was tested in other, more limited studies in Europe, the results of which have been published [30–32]. A study in Cardiff, Wales used the same protocol as the present study, but included only the 20 and 50 mcg vaccine doses with the same Alhydrogel adjuvant [30]. A limited analysis of cellular immune responses was carried out using IFN-$\gamma$ ELISpot. Immunogenicity of a single injection of ICC-1132 formulated with Seppic ISA 720 adjuvant, instead of Alhydrogel, was assessed in a Phase 1 dose response study [32] and in a Phase 1/2 trial of a single dose of 50 mcg/ISA 720 [31]. The present study was initially designed to be a phase 1/2 study of the three ICC-1132 concentrations in either saline or adsorbed to Alhydrogel, followed by a malaria challenge trial. Because the malaria challenge was not performed, this report summarizes the results of the phase 1 trial of ICC-1132 in the United States.

**Methods**

The protocol for this trial and supporting CONSORT checklist are available as supporting information; see Checklist S1 and Protocol S1.

**Participants**

The trial took place on the campuses of the University of Maryland at Baltimore and at College Park. The study methods and rationale, along with the study consent was explained to potential healthy, adult participants aged 18—45. A written exam was administered to potential participants to assess their understanding of the study procedures, rationale and expected outcomes. Consented participants were screened by medical history, physical examination and laboratory analysis of hematologic and serologic markers. Exclusion criteria included medical history or serologic indications of malaria, HIV, HBsAg or any significant cardiovascular, hepatic or renal function abnormalities.

**Interventions**

**Vaccine Construct.** The vaccine has been described [29]. In summary, recombinant ICC-1132 is comprised of the assembly domain of the HBc gene (amino acid residues 1—149) with the CS universal T cell epitope (T$^*$) (CS amino acid residues 326—345) fused to the HBc C-terminus following Val$_{149}$ [33] (Figure 1). The B and T cell CS repeat epitopes, T1 (NANPNVDPNANP) and B (NANP)$_3$, are inserted into the HBc immunodominant loop between amino acid residues Asp$_{78}$ and Pro$_{79}$. Recombinant HBc dimers self-assemble into an icosahedral virus-like particle of approximately 30 nm in diameter composed of 180—240 individual copies of the recombinant protein [29,33]. Based on HBc structure, the CS repeats contained in ICC-1132, inserted between amino acid residues 78 and 82 of HBc, are believed to be localized to the tip of surface spikes on the particle formed by dimerization of HBc monomers [33,34]. The T$^*$ epitope replaces the HBc protamine-like domain (HBc amino acid residues 150—183) and is therefore most likely oriented to the inner surface of the core particle, which is believed to be similar to its orientation in the native circumsporozoite protein.

ICC-1132 was adsorbed to aluminum hydroxide (Alhydrogel; Superfos, Frederikssund, Denmark) with >95% adsorption as determined by measurement of residual unbound protein. Each 1 mL of vaccine contained 1 mg of aluminum as Al(OH)$_3$. The saline formulation contained the same concentration of ICC-1132.
but did not contain Alhydrogel. The vaccine was produced, purified and formulated by Apovia, Inc.

**Study Design.** The study was originally designed as a double-masked, dose-escalating trial comparing the three dose levels (10, 20 and 50 mcg) of ICC-1132 in saline to the same three dose levels of ICC-1132 adsorbed to Alhydrogel. In January 2003 the trial was delayed because the saline formulation of the vaccine was found to be unstable after six to nine months storage at 4°C. The saline formulation was subsequently removed from the study, leaving the stable alum preparation to be tested. The study was redesigned to assess the 20 and 50 mcg doses of the Alhydrogel vaccine in a non-masked, dose escalating fashion. The study was conducted according to GCP guidelines. Ethical approval for the study was obtained from the Institutional Review Boards of the University of Maryland, Baltimore and University of Maryland, College Park and the New York University School of Medicine.

**Objectives**

The primary objectives were (1) to compare the safety and reactogenicity of ICC-1132 in saline and with the Alhydrogel formulation in healthy, malaria-naive human adults, and (2) to assess the immunogenicity of the two ICC-1132 vaccine formulations. A secondary objective was to vaccinate a sufficient number of volunteers who would agree to participate in a concurrent malaria challenge trial. The immunogenicity data for the saline formulation is abbreviated for reasons described. The malaria challenge trial was cancelled because of lack of sufficient immunogenicity, predefined as a dose cohort median IFA of ≥1500.

**Outcomes**

**Safety and Reactogenicity.** Local and systemic reactions were assessed for 30 minutes following each injection. Clinical assessments were carried out at 1, 2, 7, 14, 28 and 56 days after each injection, at day 84 after the second injection, and day 168 after the third injection. Telephone interviews were conducted at 4 days after each injection and volunteers maintained a daily diary to collect adverse events and twice daily body temperature recordings in the seven days immediately after each injection.

**Local Reactions.** Tenderness, pain, erythema, induration and pruritus, at the site of injection, were graded.

**Systemic Reactions.** Solicited systemic variables evaluated included fever, chills, malaise, headache, photophobia, anorexia, nausea, vomiting, abdominal pain, myalgia, arthralgia and rash. Non-solicited adverse event reported by a volunteer was recorded and an assessment of causality was performed by the investigators.

**Local and Systemic Events Grading.** Local and systemic adverse events were graded according to the following schema:

Grade 1 mild, no change in activity and/or no medication necessary; Grade 2 moderate, requires change in activity and/or medication; Grade 3 severe, bed rest required/inability to perform normal activities and/or medical intervention other than medication alone (such as an outpatient visit in emergency department or clinic, excluding hospitalization).

**Clinical Chemistry.** Urinalysis, hematological and biochemical safety analysis was carried out prior to vaccine injections, 2, 14, 28 days after all injections, 84 days after the second injection, and 36 and 168 days after the third injection.

**Immunogenicity — Serologic Assays.** Serum samples for serological assays and peripheral blood mononuclear cells (PBMC) for cellular immune assays were obtained at the time of each immunization, 14 and 28 days after each injection, 84 days after the second injection, and 36 and 168 days after the third injection. Methods used for ELISA, IFA and CSP reactions are identical to those previously published [30]. Significant values were taken to be a ≥4-fold increase from baseline titers. Antibody reactivity with viable sporozoites was assessed by circumsporozoite precipitin (CSP) reaction in which antibody mediated cross linking of surface CS protein results in formation of a terminal precipitin reaction on sporozoites detectable by phase microscopy [35]. The presence of neutralizing antibodies in immune sera was determined using a Transgenic Sporozoite Neutralization Assay (TSNA) based on transgenic rodent *Plasmodium berghei* sporozoites expressing *P. falciparum* CS repeats [36,37]. In the TSNA, 2x10⁹ transgenic sporozoites were incubated in serum (1:5 dilution) from each volunteer or with controls of medium only or 25 mcg/mL of either mAb 3D11, specific for *P. berghei* repeats, or mAb 2A10, specific for *P. falciparum* repeats. Sporozoites were incubated with serum, mAb or medium for 40 minutes on ice prior to their being added to human hepatoma cell (HepG2) cultures. The cultures were incubated for 72 hours followed by extraction of total RNA from the cells for determination of intracellular parasites by RT-PCR using primers specific for parasite 18S ribosomal RNA. All TSNA were performed in duplicate. Inhibition of >85% is considered positive.

**Immunogenicity — Cellular Assays.** The PBMC were Ficoll purified from blood collected in citrate buffer Vacutainer (BD Biosciences, San Diego, CA). Short term TCL were used in the assays by expanding PBMC (2x10⁶/mL) with a single in vitro stimulation with rCS (10 mcg/mL) with recombinant human interleukin-2 (IL-2) added on day five [32,38]. In the proliferation assay, the short term TCL were incubated in triplicate wells with various concentrations of rCS or HBc proteins or peptides representing the CS repeats (T1B) or the universal T cell epitope (T*). Culture wells titrated with 3H-Tdr on day five were incubated overnight and harvested. The results are expressed as delta (d) cpm (cpm in cultures stimulated with antigen – cpm in cultures without antigen) or stimulation index (SI) (cpm in antigen stimulated cultures/cpm induced by supernatants from medium only cultures). IL-2 was measured in a bioassay using 24 hour cell culture supernatants incubated with an IL-2 dependent cell line [32,38] and the results expressed as d cpm or SI. Significant responses were taken as d cpm > mean ± 2 standard deviations of responses obtained with pre-immune cells from the volunteers.

**Sample size**

In this phase 1 descriptive study focusing on safety and immunogenicity, sample sizes were derived from logistic considerations, rather than by power analyses. The larger number of volunteers in the 50 mcg cohort was designed to yield sufficient interested volunteers to enable a malaria challenge trial after the third vaccination.

**Randomization—Sequence generation**

Using SAS, a randomization list was prepared in advance of vaccination activities with randomized block of *N=2* (one subject receiving saline and the other Alhydrogel formulated vaccine) for the August 2002 cohorts.

**Randomization—Allocation concealment**

Assignments to vaccine groups from this randomization were placed in individual sealed envelopes and were provided to the immunization team. Randomization assignment was masked from investigators assessing post-injection adverse events.

**Randomization—Implementation**

The original study statistician generated the allocation sequence. Subjects in the 10 mcg cohort and the first three
subjects in the 20 mcg cohort were randomized by computer to receive ICC-1132 in saline or ICC-1132 adsorbed to Alhydrogel. All 32 subsequent subjects received ICC-1132 adsorbed to Alhydrogel, and were allocation to either the 20 mcg or 50 mcg cohorts on a first come, first serve basis, such that the first eight available subjects were assigned the 20 mcg cohort, while the last 24 subjects were assigned the 50 mcg cohort.

**Blinding**

The participants, vaccine administrators and clinical staff providing safety follow-up were blinded to which formulation of vaccine subjects had received prior to removal of the saline formulated vaccine from the study. Neither the subjects nor investigators were blinded once the saline formulation was removed from the trial, because only one formulation was used and because the dose-escalation design required that the safety of the 20 mcg dose be evaluated prior to administration of the 50 mcg dose.

**Statistical methods**

Quantitative data were assessed for normal distribution and log-transformation was performed where appropriate. Differences among proportions were compared using Fisher’s exact tests, differences between medians were compared using the Wilcoxon Rank Sum test and Spearman’s Rank Correlation test was used to assess association strengths. All tests were two sided. A \( P \leq 0.05 \) was taken to be statistically significant. R v2.2.0 [39] was used for statistical analysis.

**Results**

**Participant Flow and Recruitment**

A total of 51 volunteers received at least one injection and 29 volunteers completed the study to receive all three injections of ICC-1132 adjuvanted to Alhydrogel (Figure 2).

**Baseline demographic data**

The distribution of volunteers by gender and ethnicity was similar statistically amongst all dose and formulation groups \( (P=0.76) \). The median age of all trial participants was 25 years, with a range of 18—45 years. There was a statistically significant difference in participant age between the Baltimore (median age 26.5 [24 to 31]) and College Park (median age 21 [21 to 25]) cohorts \( (P=0.008) \).

**Safety**

The vaccine was well tolerated at the dose levels examined in this study. No severe (grade 3) adverse events occurred, nor any clinically significant laboratory abnormalities attributable to...
vaccination. One serious adverse event occurred, unrelated to
vaccination, in a volunteer who became pregnant within three
months of receiving the third 20 mcg injection. She delivered a
healthy infant at 40 weeks. No significant differences in reactoge-
nicity were noted between dose cohorts, between the first, second
and third vaccination, nor between Alhydrogel and saline vaccine
groups.

Local Reactogenicity. Of 125 total vaccinations admini-
stered to 51 volunteers, local adverse events were principally
limited to mild tenderness or pain at the injection site in 46
volunteers. In addition, four volunteers had local pruritus and one
volunteer each had an episode of induration and erythema
(Table 1). Most reactions were mild and lasted 1—3 days. There
were no severe reactions.

Systemic Reactogenicity. Headache and myalgia were the
most common manifestations of systemic reactogenicity, followed
by other elicited symptoms (Table 2). Symptoms were generally
mild and persisted 1—5 days. There were no severe or serious
reactions. Because no placebo group was included it is difficult to
ascrue causality to the vaccine. No delayed reactogenicity was
reported by the 28 volunteers who were contacted on day 336
after the third vaccination (168 days after the third vaccination).

Immunogenicity

Humoral Immune Responses. Depending upon the
cache dose, 95—100% of volunteers developed specific
responses to the ICC-1132 vaccine immunogen (Table 3). How-
ever, only 50—75% of the volunteers developed (T1B)4
malaria specific responses, while 29—63% developed IFA malaria
specific responses. There was no significant boosting of malaria
specific humoral immune responses [(T1B)4 and IFA] after the
third injection of ICC-1132 (Table 3). Less than a four-fold
boosting of anti-HBc titers occurred after the third injection in
both the 20 and 50 mcg cohorts. This increase in anti-HBc titer
was statistically significant in the 50 mcg cohort (P = 0.02).

The anti-ICC-1132 and anti-HBc GMTs were significantly
higher in the 10 mcg cohort compared to the 20 and 50 mcg
cohorts after the second injection, although the percent responders
to the two antigens (90—100%) were nearly identical in the three
cohorts (Table 3). The 10 mcg cohort was not vaccinated a third
time. The anti-ICC-1132 and anti-HBc GMTs and percent
responders in the 20 and 50 mcg cohorts after each of three
injections were similar.

Malaria specific anti-(T1B)4 response was significantly higher
in the 10 mcg cohort compared to the 50 mcg cohort after the
second injection (P = 0.019) (Table 3). Although the IFA GMT
and percent responders were higher in the 10 mcg as compared to
the 20 and 50 mcg cohorts, the differences were not statistically
significant. Overall, the malaria specific responses were low in
comparison to immunogen specific responses.

Anti-ICC-1132, anti-HBc and anti-(T1B)4 IgG1 and IgG3
subtypes, typical of Th1-type immune responses, developed
preferentially over IgG2 and IgG4 subtypes (P = 3.33−5) without
affect of the adjuvant (P = 0.21) (titers expressed in O.D.; data not
shown).

Two (volunteers 1 and 10) of the seven volunteers in the 10
mcg alum cohort (those with the most robust malaria specific
humoral responses) developed positive CSP reactions, which
demonstrates the presence of specific antibodies capable of cross-
linking surface CS protein on the viable sporozoite. Inhibition in
the TSNA using PfPb sporozoite invasion of HepG2 cells ranged
from a 54% inhibition (volunteer 3) to a 49% increase (volunteer
1) in 18S rRNA copies at 28 days post-vaccination two (10 mcg
cohort) or 28 days post-vaccination three (20 mcg cohort)
(Figure 3). TSNA data for the 50 mcg cohort is not available.

There was a correlation between IFA titer and CSP reactions,
though only two volunteers in the 10 mcg cohort had positive
CSP reactions (ρ = 0.70, P = 0.003). The two highest responders
by IFA and those with the positive CSP reactions, volunteers 1
and 10, demonstrated little to no inhibition by the TSNA
(Figure 3).

Cellular Immune Responses. Depending on vaccine dose,
65—75% of the volunteers’ demonstrated T cell proliferation in
response to ICC-1132 and 47—75% had proliferation to rCS.
The magnitude of responses to these two antigens was similar
(Table 4). Seven of 28 volunteers gave a T cell response to the
universal T cell epitope (T*)4, as measured by IL-2 bioassays
(Table 5). Only one volunteer had a consistent response to the
restricted CS repeat (T1B)4. Fifty-six days after the third injection
of the ICC-1132 vaccine no volunteer had significant cellular
immune responses in either the proliferation or the IL-2 bioassay
(data not shown). There was good correlation between the
proliferation assays and IL-2 bioassays (ρ = 0.5—0.9, for
individual cohorts), though the P values for the 10 mcg cohort
correlations were >0.05.

Discussion

Interpretation

This P. falciparum CS-based malaria vaccine, a novel virus-like
particle, was safe and well tolerated. It elicited robust antibody
responses to the immunogen (ICC-1132) and to HBc, but the
malaria specific antibody responses were relatively weak. For
example, 95—100% of all volunteers developed specific responses to the ICC-1132 vaccine immunogen and to HBc, but only 50—75% developed anti-CS repeat responses and 29—63% developed IFA sporozoite responses (Table 3). The geometric mean titers by IFA and rCS ELISA were suboptimal, arbitrarily defining an IFA titer of 1500 as minimal adequate response for malaria challenge, and only weak boosting was seen after the third vaccination. In cellular responses, 8 of 28 (29%) and 7 of 28 (25%) responded to rCS and the universal T cell epitope (T*)4 malarial antigens respectively (Table 5). Because only a limited number of class II genotypes can function as restriction elements for the CS repeat epitope, it was not unexpected to find only 1 of 28 persons consistently respond to the HLA-restricted (T1B)4 antigen (Table 5). Although the cellular proliferation response to the ICC-1132 and rCS antigens were similar, this candidate malaria vaccine did not achieve our predefined criteria, a median IFA response of 1:1500, to justify a malaria challenge.

Table 3. GMT of All Vaccinees (Percent Responders) Against Immunogen (ICC-1132), Hepatitis Core (HBc), Malaria Repeat Antigen ((T1B)4) and Whole Sporozoite (IFA)

| Antigen         | ICC-1132* | HBc* | (T1B)* | IFA† |
|-----------------|-----------|------|--------|------|
| Post Dose       | 1         | 2    | 3      | 1    | 2    | 3    | 1    | 2    | 3    |
| 10 μg           | 320       | 16255|| NA  | 63   | 4561*|| NA  | 67   | 269**| NA  | NA  | 226  | NA  |
| (88)            | (100)     | (33) | (100)  | -    | (25) | (75) | -    | (63) | -    |    |
| 20 μg           | 123       | 905* | 3121   | 73   | 905* | 2319 | <80  | 106  | 101  | NA  | 80  | 66   |
| (50)            | (100)     | (100)| (100)  | (100)| (100)| (13) | (50) | (43) | (25) | (29) |
| 50 μg           | 88        | 10043| 2661   | 49   | 577*| 2463 | 45   | 63** | 138  | NA  | 57  | 118  |
| (48)            | (95)      | (94) | (17)   | (90) | (94) | (8)  | (8)  | (38) | (53) | -   | (25) | (53) |

**ELISA
1Indirect immunofluorescence assay
2P=0.008, between ICC-1132 10 and 20 μg cohorts post dose 2
3P=0.001, between ICC-1132 10 and 50 μg cohorts post dose 2
4P=0.03, between HBc 10 and 20 μg cohorts post dose 2
5P=0.007, between HBc 10 and 50 μg cohorts post dose 2
6P=0.019, between (T1B)4 10 and 50 μg cohorts post dose 2
7Differences between 10, 20 and 50 μg cohort percent responders not statistically significant

Vaccination on days 0, 56 and 168
NA = Not available
doi:10.1371/journal.pone.0001556.t003

Figure 3. Comparison of Antibody Titer and Sporozoite Neutralizing Activity for Select Volunteers. As measured by IFA against whole sporozoite (gradient bars), (T1B)4 ELISA (hatched bars) and transgenic sporozoite neutralization assay (TSNA) (percentages above bars). For the TSNA, 94.5% inhibition was obtained using a positive control monoclonal antibody specific for P. falciparum CS repeats and 25% for negative control MAB specific for P. berghei CS repeats. Volunteers with positive CSP assays, volunteers 1 and 10, are indicated with a plus sign (+) in the x-axis.
doi:10.1371/journal.pone.0001556.g003

Table 4. TCL Proliferation Median δCPM (Percent Responders) All Vaccinees Days 84 and 196

| Cohort      | Day 0 | Day 84 | Day 196 |
|-------------|-------|--------|---------|
|             | ICC-1132 rCS | ICC-1132 rCS | ICC-1132 rCS |
| 10 μg*      | 3033  | 4459   | NA      | NA      |
| -           | -     | (75)   | (75)    | -       |
| 20 μg†      | 587   | 3484   | 15903   | 4736    | 10451   | 13772  |
| -           | -     | (67)   | (75)    | (57)    | (29)    |
| 50 μg       | 310   | 462    | 3636‡   | 11948‡  | 10035|| 11063||
| -           | -     | (13)   | (29)    | (65)    | (47)    |

1Data from 4 volunteers
2Data from 7 volunteers
3Responses 14 days after the second dose of ICC-1132 (Day 70)
4Data from 8 volunteers
5Data from 14 volunteers
6Data from 17 volunteers
7Differences between 10, 20 and 50 μg cohorts δCPM & percent responders not statistically significant

Vaccination on days 0, 56 and 168
NA = Not available
doi:10.1371/journal.pone.0001556.t004
The predominant IgG1 and IgG3 was noted over IgG2 and IgG4 as found in previous studies of malaria-specific responses. The vaccine lots differed without precedent that a lower immunogen dose would lead to a more robust humoral response [40–42]. The vaccine lots differed in the manner in which antigen bound to the adjuvant in the second injection. Intercohort differences may have been an effect of the impact in which antigen bound to the adjuvant in the lower versus higher dose vaccines. Although unusual, it is not without precedent that a lower immunogen dose would lead to a more robust humoral response [40–42]. The vaccine lots differed between the 10 mcg and the 20 and 50 mcg cohorts because of the delay in the trial. The 10 mcg vaccine lot in our study was the same as was used in the Cardiff study of 20 and 50 mcg cohorts [30] so it is helpful to compare these results with our own. There remains a statistically significant difference between our 10 mcg and the Cardiff 20 and 50 mcg cohorts when comparing ICC-1132 GMT at day 84 (GMT 989 and 1522 in the Cardiff 20 and 50 mcg cohorts respectively) (P<0.035 in all cases), but there were no statistically significant differences in the percent responders.

**Hepatitis B Core Effects.** Volunteers with preexisting anti-HBc titers (two volunteers in the 10 alum and one volunteer in the 50 mcg cohort) demonstrated more robust humoral responses to immunogen and HBc, with titers consistently higher than their respective cohorts’ GMT. There was not a noticeably increased response in these individuals against malaria-specific antigens. This suggests that, in these previously anti-HBc positive individuals, the more robust response against the immunogen was specific for HBc epitopes. Indeed, the higher antibody titers seen against both the immunogen and HBc in all the volunteers could imply that the targeted epitopes in the immunogen are shared HBc epitopes, rather than malaria epitopes. The proliferation data shows that the cellular immune response is targeting malaria-specific responses, as the median δCPM against rCS and ICC-1132 are similar at day 196 (Table 4). As has been demonstrated in other studies, the cellular immune response against *P. falciparum* sporozoites is likely a critical component of protection [7,43,44]. Analysis of cytokine and IFN ELISpot will be reported in a separate paper (C. Othoro, manuscript in preparation).

**Th1 Type Response.** Volunteers developed Th1-type antibody, with anti-CS IgG1 and IgG3 subtypes developing preferentially over IgG2 and IgG4, as found in previous study of this vaccine [30]. This effect was independent of the adjuvant, as similar predominance of IgG1 and IgG3 was noted in the saline group (data not shown), suggesting a Th1 inducing property of the core particle [43]. The practical effect of a preferential Th1-type antibody response would be production of gamma interferon, a known inhibitor of hepatic stage intracellular parasites [43,46,47].

**Functional Assays.** The good correlation between (T1B)4 ELISA and IFA titers suggests that anti-repeat antibodies elicited by the ICC-1132 vaccine recognize native CS on the sporozoite (Figure 3). Two individuals with the highest IFA titers gave a positive CSP reaction [30]. There was no correlation between either IFA or CSP reaction and level of inhibition in the TSNA using PfPb sporozoite invasion of HepG2 cells (Figure 3). It is possible that our antibody titers and/or avidity were not sufficient to lead to inhibition in the TSNA. In a previous study, antibodies developed against a (T1B)4 MAP [27] vaccine demonstrated high level inhibition of invasion of HepG2 cells in the TSNA [37], however challenge studies were not carried out in that study and correlation with protection in vivo remains unknown. In malaria blood stage vaccines, functional assays based on transgenic parasites expressing falciparum blood stage epitopes were more predictive of protection, presumably because the ELISA does not measure functional antibodies [48,49]. As yet there remains no accurate correlate of protection for malaria sporozoite challenge [15,18,50,51]. Previous studies have attempted to correlate protection and inhibition of invasion of *P. falciparum* sporozoites into hepatoma cells (TSI) assay [52–56]. Still others have suggested the use of IFN-γ ELISpot to measure T cell responses or opsonizing anti-CS antibodies as correlates of protection [7,57]. Proper correlates of protection may well vary depending upon the antigen/adjuvant combination tested.

**Comparison to Other Studies of Same Immunogen.** After initiation of this present study in the USA, the vaccine was tested in other, more limited studies in Europe, the results of which have been published [30–32]. One study assessed immunogenicity of a single injection of 5, 20 or 50 mcg of ICC-1132 formulated with the more potent adjuvant, Seppic ISA 720, instead of Alhydrogel [32]. A single dose of this 50 mcg ICC-1132/ISA 720 elicited maximal GMT anti-repeat ELISA titers of 1050 (100% seroconversion), higher than in the current study with maximal anti-(T1B)4 GMT of 269 (75% seroconversion). In contrast, our alum formulation elicited peak anti-HBc responses that were more robust, exceeding 16000 GMT following booster immunization, as compared to peak anti-HBc GMT of 830 elicited by a single immunization with the ISA 720 formulation. In a second study of the ISA 720 formulation, 9 of 11 volunteers receiving a single 50 mcg injection of ICC-1132/ISA 720 developed positive anti-repeat antibody responses (GMT 370) but minimal T cell IFN ELISpot responses [31]. Following challenge with *P. falciparum* sporozoite, there was no appreciable effect of vaccination on malaria infection, indicating that multiple immunization and/or higher antibody titers or more robust cellular immune responses are required for protection.

In summary, the candidate vaccine, ICC-1132, was safe and well-tolerated at all dose levels examined in this trial. ICC-1132 was poorly immunogenic when adjuvanted with alum. The immunogenicity of the candidate vaccine may be improved through combination with a more potent adjuvant.

**Supporting Information**

**Checklist S1** CONSORT Checklist
Found at: doi:10.1371/journal.pone.0001556.s001 (0.10 MB PDF)

**Protocol S1** Final Trial Protocol

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**Table 5.** Fine Specificity of Malaria Specific Responses: Median IL-2 CPM All Vaccinees (Number of Responders/Number of Total, as measured by δCPM)

| Cohort | Antigen | (T*)4a | (T1B)4a |
|--------|---------|--------|---------|
| 10 µg† | 12114 (0/4) | 47 (1/4) | 52 (0/4) |
| 20 µg‡ | 1515 (1/7) | 268 (2/7) | 242 (1/7) |
| 50 µg§ | 2831 (7/17) | 147 (4/17) | 55 (0/17) |
| Total Percent Responders | 29 (8/28) | 25 (7/28) | 4 (1/28) |

† T universal epitope
‡ Responses 14 days after the second dose of ICC 1132 (Day 70)
§ Responses 28 days after the third dose of ICC 1132 (Day 196)

Differences between cohorts not statistically significant.

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Author Contributions

Conceived and designed the experiments: EN AG RE. Performed the experiments: EN AG RE GO CO JC. Analyzed the data: EN AG RE GO CO JC. Contributed reagents/materials/analysis tools: EN GT. Wrote the paper: EN AG RE.
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