Safety and efficacy after immunization with Plasmodium falciparum sporozoites in the controlled human malaria infection model

Schats, R.

Chad. April 2005. Team refugee camp Forchana

CHAPTER 2

Cytotoxic markers associate with protection against malaria in human volunteers immunized with *Plasmodium falciparum* sporozoites

Else M. Bijker*, Anne C. Teirlinck*, Remko Schats, Geert-Jan van Gemert, Marga van de Vegte-Bolmer, Lisette van Lieshout, Joanna IntHout, Cornelus C. Hermsen, Anja Scholzen, Leo G. Visser, Robert W. Sauerwein.

*Contributed equally

The Journal of Infectious Diseases. 2014 Nov 15;210(10):1605-15.

ABSTRACT

Background

Immunization of healthy volunteers under chloroquine chemoprophylaxis by bites from *Plasmodium falciparum (Pf)*-infected mosquitoes (CPS immunization) induces sterile protection against malaria. CPS-induced protection is mediated by immunity against pre-erythrocytic stages, presumably at least partially by cytotoxic cellular responses. We therefore aimed to investigate the association of CPS-induced cytotoxic T cell markers with protection.

Methods

In a double-blind randomized controlled trial (NCT01218893), we performed dose titration of CPS immunization followed by homologous challenge infection in 29 subjects. Immune responses were assessed by *in vitro* restimulation of PBMCs and flow cytometry.

Results

Dose-dependent complete protection was obtained in 4/5 volunteers after immunization with bites from a total of 45, in 8/9 volunteers with 30, and in 5/10 volunteers with 15 *Pf*-infected mosquitoes respectively (OR=5.0; 95% CI 1.5-17). Proportions of CD4 T cells expressing the degranulation marker CD107a and CD8 cells producing granzyme B after *Pf*-restimulation were significantly higher in completely protected subjects (OR=8.4; 95% CI 1.5-123; p=0.011 and OR=11; 95% CI 1.9-212; p=0.004 respectively).

Conclusions

These data underline the efficiency of CPS immunization to induce sterile protection, and support a possible role for cytotoxic CD4 and CD8 T cell responses in pre-erythrocytic immunity.

INTRODUCTION

Malaria remains a major public health problem, with an estimated incidence of 207 million clinical cases leading to approximately 627,000 deaths every year (1). *Plasmodium falciparum (Pf)* is the most severe and lethal of five species that can cause malaria in humans. Availability of an effective vaccine will be critical to fight this disease, but currently there is no licensed vaccine available, despite decades of research. Most efforts have focused on the development of subunit vaccines, unfortunately showing only limited protective efficacy (2, 3). Immunization strategies based on whole parasites, however, have repeatedly induced high levels of protection in experimental settings (4-7). Previously we showed that immunization of healthy, malaria-naive subjects, while taking chloroquine chemoprophylaxis, with live sporozoites delivered by 36-45 mosquito bites (ChemoProphylaxis and Sporozoites (CPS) immunization) induces robust, long-lasting sterile protection against *Pf* malaria (8, 9). CPS immunization is about 20 times more efficient than the only alternative approach for complete sterile protection against malaria in humans i.e. immunization with radiation-attenuated *Pf* sporozoites (RAS), requiring bites from >1000 infected and irradiated mosquitoes (4), or intravenous administration of 675,000 sporozoites (10).

CPS-induced protective immunity targets the earliest stages of the parasite lifecycle, i.e. sporozoites and/or liver stages, rather than the subsequently developing asexual blood stages (11). The immune pathways responsible for this pre-erythrocytic protection, however, remain unknown. In murine malaria models, cytotoxic killing of *Plasmodium*-infected hepatocytes appears to play a role in protection, but the exact contribution and mechanism of cytotoxicity remain elusive (12, 13). Also in humans, a role for both cytotoxic CD4 T cells and CD8 T cells has been suggested, but evidence is scarce and largely circumstantial (reviewed by Tsuji et al. (14)). We conducted a double-blind randomized controlled CPS immunization dose titration and challenge study. Subjects, while taking chloroquine prophylaxis, were immunized by bites from a total of 45 (3x15), 30 (3x10) or 15 (3x5) infected mosquitoes followed by a challenge infection, resulting in dose-dependent protection. Next, we explored markers of cytotoxic T cell responses induced by CPS immunization and identified two cytotoxic markers associated with protection.

MATERIALS AND METHODS

Human ethics statement

All subjects provided written informed consent before screening. The study was approved by the Central Committee for Research Involving Human Subjects of The Netherlands (NL33904.091.10) and complied with the Declaration of Helsinki and Good Clinical Practice including monitoring of data. ClinicalTrials.gov Identifier: NCT01218893.

Clinical trial design and procedures

A single centre, double-blind study was conducted at the Leiden University Medical Center from April 2011 until April 2012. Healthy subjects between 18 and 35 years of age with no history of malaria were screened as described previously (11). Thirty subjects were randomly divided into four groups using a computer-generated random-number table. Subjects, investigators and primary outcome assessors were blinded to the allocation. All subjects received CPS immunization as described previously (8, 11), but the number of NF54 *Pf* infected versus uninfected mosquitoes varied per group: five subjects received three times bites from 15 infected mosquitoes (Group 1), ten subjects received three times bites from 10 infected and 5 uninfected mosquitoes (Group 2), ten subjects received three times bites from 5 infected and 10 uninfected mosquitoes (Group 3) and five control subjects received three times bites from 15 uninfected mosquitoes (Group 4). Nineteen weeks after the last immunization (fifteen weeks after the last chloroquine dose), all subjects were challenged by the bites of five mosquitoes infected with the homologous NF54 *Pf* strain, according to previous protocols (8, 15). The primary outcome was prepatent period, defined as the time between challenge and first positive thick blood smear. Thick blood smears were prepared and read as described previously (11). For more details about the immunization and challenge procedures and follow-up, see supplementary information.

Immunological methods

Peripheral blood mononuclear cells (PBMCs) were collected on the following time points: before initiation of chloroquine prophylaxis (baseline; B), 27 days after each immunization; I1, I2 and I3 (I1 and I2 are one day before the second and third immunization respectively), the day before and twenty weeks after the challenge infection (C-1 and C+140). For the assessment of *Pf* specific immune responses, PBMCs were restimulated *in vitro* with *Pf* infected red blood cells (*Pf*RBC) as described before (16). Expression of the degranulation marker CD107a, the cytotoxic molecule granzyme B and the cytokine IFNγ by CD4, CD8 and γδ T cells was assessed by flow cytometry. For a detailed description, see supplementary information.

Statistical analysis

The dose-dependent induction of protection was tested by logistic regression using SPSS 20. Comparison of CD107a expression and granzyme B and IFNγ production by T cell subsets between immunized unprotected and protected volunteers after CPS immunization was done per selected cellular response by means of Firth's penalized logistic regression (17, 18), resulting in p-values, odds ratios (OR) related to a change of one interquartile range, and 95% profile likelihood Confidence Intervals (95% CI) for the OR, using R software version 3.0.1 (19), with R packages logistf version 1.21 (20), rms version 4.1-3 (21) and penalized version 0.9-42 (22, 23). The ability of (a combination of) markers to discriminate between protected and unprotected volunteers was assessed with the Area under the Receiver Operator Curve (ROC), based on leave-one-out cross-validation (LOOCV), using the R-software and pROC package version 1.7.1 (24). For further details, see supplementary information.

RESULTS

CPS immunization

Thirty volunteers were included (median age 21 years, range 19–31), out of sixty-three subjects screened for eligibility (**Figure 2.S1**). Volunteers were randomly assigned to four groups and received CPS immunization by bites from 3x15 (Group 1, n=5), 3x10 (Group 2, n=10) or 3x5 (Group 3, n=10) mosquitoes infected with strain NF54 sporozoites. Control subjects (Group 4, n=5) received chloroquine prophylaxis and bites from 3x15 uninfected mosquitoes. After each consecutive immunization the number of subjects with parasitemia, as retrospectively detected by qPCR, steadily decreased in Group 1 and 2. In Group 3, however, five volunteers still showed parasitemia after the second and third immunization (**Figure 2.1**). Remarkably, in four immunized subjects, parasitemia was never detectable by qPCR at any time point (three subjects in Group 2, one in Group 3). One subject from Group 2 withdrew consent after the first immunization for reasons unrelated to the trial, and was excluded from the analysis.

Challenge infection

Nineteen weeks after the last immunization, volunteers were challenged by standard exposure to bites from five homologous strain NF54-infected mosquitoes (5). Protection by CPS immunization was dose-dependently induced in four out of five subjects in Group 1, eight out of nine subjects in Group 2 and five out of ten subjects in Group 3, while all control subjects became thick smear positive (OR=5.0; 95% CI 1.5-17; p=0.01). The median prepatent period was 2.5 days longer in CPS-immunized unprotected subjects compared to controls, both by thick smear and qPCR. Although not statistically significant (p=0.22 and 0.31 respectively), this delay is suggestive for the presence of partial protection at least in some of the unprotected CPS-immunized subjects (**Figure 2.2** and **Table 2.1**). In retrospect, all six volunteers with detectable parasitemia by qPCR after the third immunization were not completely protected from challenge infection, while 17 out of 18 subjects with a negative qPCR after the third immunization were fully protected.

Platelets decreased below reference value ($150x10^9$/L) in eight out of twelve thick smear positive (TS+; i.e. both controls and CPS-unprotected) subjects at any point after challenge (median for all TS+: $134x10^9$/L, range 79 - $213x10^9$/L). D-dimer was elevated in all TS+ subjects after challenge (median

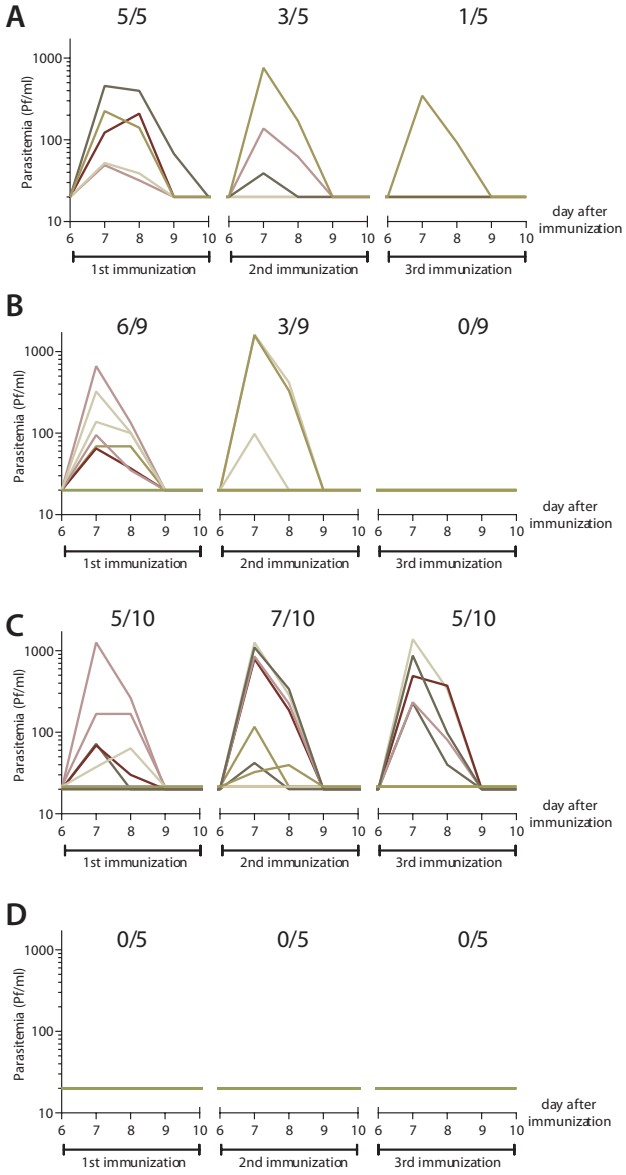

Figure 2.1 Parasitemia after the first, second and third CPS immunization.
*Parasitemia was determined once daily by qPCR from day 6 until day 10 after each immunization. Each line represents an individual subject. Panels show data for volunteers from (**A**) Group 1 (3x15), (**B**) Group 2 (3x10), (**C**) Group 3 (3x5) and (**D**) Group 4 (controls). Values shown as 10 on the log-scale were negative (i.e. half the detection limit of the qPCR: 20 parasites/ml). The number of subjects with a positive qPCR/total number of volunteers after each immunization are shown below the graphs.*

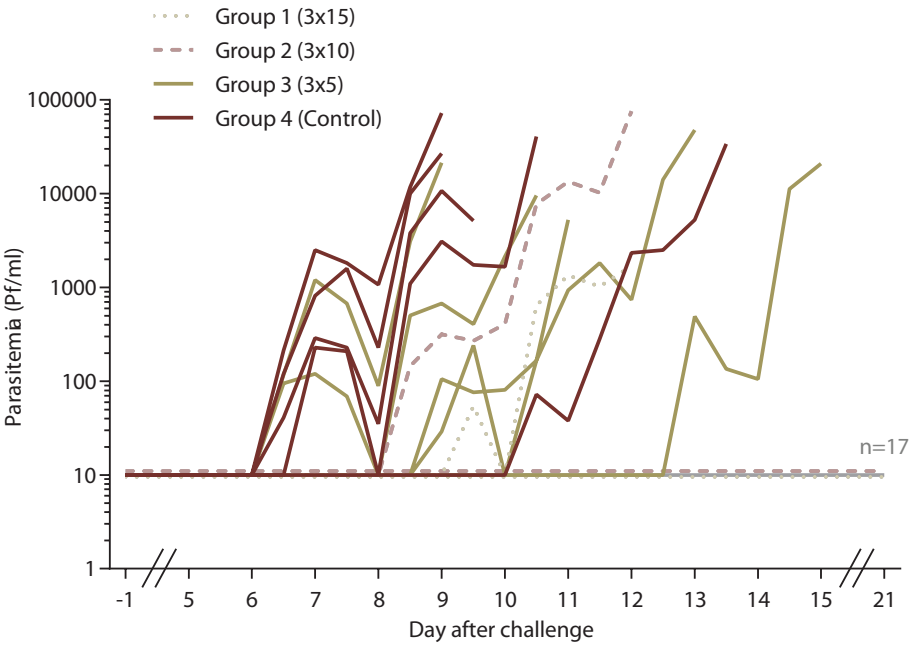

Figure 2.2 Parasitemia after challenge infection.
*Parasitemia was assessed retrospectively by real-time quantitative PCR (qPCR) from day 5 after challenge onwards, up until day 21, at two time points per day for TS+ volunteers, and one time point per day for protected volunteers. Each line represents an individual subject. **Brown** dotted lines show CPS-immunized volunteers from group 1 (3x15; n=5), **red** dashed lines subjects from group 2 (3x10; n=9), **brown** solid lines subjects from group 3 (3x5; n=10) and **red** lines represent malaria-naive control subjects (n=5). Values shown as 10 on the log-scale were negative. The two TS+ subjects from Group 1 and 2 became qPCR positive on day 8.5 and 9.5 respectively, both became thick smear positive on day 12.0.*

Table 2.1 Protection against challenge infection after CPS immunization.

Group (# of Pf-infected mosquitoes used for immunization)	Protection		Day of positivity after challenge (TS+ subjects)[c]	
	Number[a]	Percentage[b]	Thick smear	qPCR
Group 1 (3x15)	4/5	80 (36.0 – 98.0)	12.0	9.5
Group 2 (3x10)	8/9	89 (54.3 – >99.9)	12.0	8.5
Group 3 (3x5)	5/10	50 (23.7 – 76.3)	11.0 (9.0-15.0)	9.0 (6.5-13.0)
Group 4 (Control)	0/5	0 (0.0 – 48.9)	9.5 (9.0-13.5)	6.5 (6.5-10.5)

[a] *Presented as protected/total number of subjects*
[b] *Presented as % protected (95% CI by modified Wald Method)*
[c] *Presented as median (min-max).*

peak concentration 2431 ng/mL, range 1014-5000 ng/ml). Parameters normalized in all subjects after treatment without complications. All TS+ subjects experienced solicited adverse events (AEs) during challenge infection consistent with uncomplicated malaria (median number of AEs per subject 9.5 (range 4-14), median duration of each AE 1.1 days (range 0.0-12.3)). As expected, protected subjects presented with less AEs: 15 out of 17 subjects experienced solicited AEs possibly or probably related to the challenge (median number of AEs per subject: 2 (range 0-15), median duration 0.7 days (range 0.00-15.9)). One subject from Group 2 was preliminarily treated with atovaquone/proguanil at day 10.5 after challenge because of unrelated exertional rhabdomyolysis after extensive sports activity (weightlifting) followed by sauna visits. No other severe adverse events (SAE) occurred. One volunteer from Group 1 was treated for reasons unrelated to the trial at day 19. Both these volunteers remained parasite negative by qPCR analysis after the third immunization and at any time point after challenge and were considered protected in further analysis.

Analysis of cytotoxic T cell markers after in vitro Pf-stimulation

Next, we tested a panel of representative cytotoxic T cell markers including surface expression of degranulation marker CD107a, and granzyme B and IFNγ production in CD4, CD8 and γδ-T cells after *in vitro* restimulation with *Pf*-infected red blood cells (*Pf*RBC) in all immunized subjects (**Table 2.2**). CPS-immunization induced a significant increase in both the percentage and iMFI of CD107a positive CD4 and γδ-T cells, already after the first immunization up until challenge. Similarly CD8 T cells expressed a significantly higher CD107a iMFI after the second immunization. The proportion of granzyme B positive cells did not change after immunization, but granzyme B iMFI was significantly increased in both CD8 and γδ-T cells, returning to baseline at C-1. Production of IFNγ was induced in all T cell subsets, but most pronouncedly in CD4 and γδ-T cells. There were only weak correlations between cellular responses on C-1 and total blood-stage parasite exposure, as calculated by the sum of parasites/ml after all three immunizations (data not shown, Spearman's rho for all <0.5). None of the responses in the control group changed significantly from baseline at any point of time (**Table 2.2**), suggesting that chloroquine alone did not affect *P. falciparum* specific T cell responses.

We next assessed the association of these markers with protection after challenge (**Figure 2.3**). Indeed, complete protection associated with the proportion of CD107a positive CD4 T cells (OR=8.4; 95% CI 1.5-123; p=0.011, **Figure 2.3A**), the iMFI of CD107a on CD4 T cells (OR=11; 95% CI 1.6-188; p=0.011, data not shown) and granzyme B by CD8 T cells (OR=11; 95% CI 1.9-212; p=0.004,

Table 2.2 Cytotoxic T cell markers induced by CPS immunization[a,b,c]

		CD4 T cells									CD8 T cells					γδ T cells				
Marker		B	I1		I2		I3		C-1		B	I1	I2	I3	C-1	B	I1	I2	I3	C-1
CD107a	%	0,26	0,53	***	0,64	***	0,59	***	0,53	***	0,08	0,09	0,15	0,14	0,19	26,6	36,5 ***	41,2 ***	40,3 ***	33,8 **
CD107a	iMFI	15,7	38,5	***	46,0	***	41,0	***	37,0	***	7,6	14,5	19,2 *	17,5 *	20,9 **	4472	6925 ***	7925 ***	7594 ***	6262 **
Granzyme B	%	0,25	0,49		0,41		0,38		0,43		0,50	0,89	0,92	1,03	0,94	6,06	7,35	8,27	8,96	8,79
Granzyme B	iMFI	1,40	9,80		11,3		7,50		3,24		5,11	71,3 *	40,5	26,7	19,9	2,54	15,3 ***	15,0 ***	12,4 ***	8,18
IFNγ	%	0,08	0,43	***	0,54	***	0,42	***	0,47	***	0,04	0,11	0,13 *	0,08	0,13 **	6,01	13,6 ***	16,3 ***	14,9 ***	12,0 **
IFNγ	iMFI	5,16	32,2	***	37,7	***	25,7	*	37,2	***	2,96	9,8	9,3	6,4	12,5 **	407	1020 **	1173 ***	1029 **	982 *

[a] *PfRBC-specific responses were corrected for uRBC background, mean responses for all immunized volunteers (n=24) are shown. B= baseline; I= 27 days after indicated immunization; C-1=one day before challenge.*

[b] * *=p<0.05, **=p<0.01, ***=p<0.001 (one-way ANOVA; Dunnett's post hoc test, with B as control column)*

[c] *iMFI = integrated geometric mean fluorescence intensity (percentage positive cells multiplied by the MFI of this positive population).*

Figure 2.3E) at C-1. A subgroup analysis of data from Group 3 only confirmed these findings: the proportion of both CD107a positive CD4 T cells and granzyme B positive CD8 T cells were the only markers higher in protected subjects (OR=4.2; 95% CI 0.9-140; p=0.081 and OR=27; 95% CI 1.5-27687; p= 0.019 respectively). While expression of CD107 on CD4 T cells and granzyme B in CD8 T cells predicted protection with an Area Under the ROC Curve (AUC) of 0.73 (95% CI 0.48-0.98) and 0.81 (95% CI 0.63-0.99) respectively, combining both markers resulted in only a slight improvement of the AUC (0.82, 95% CI 0.61-1).

Pf-specific IFNγ production by CD4, CD8 or γδ-T cells could not distinguish protected volunteers (**Figure 2.3G, 3H** and **3I**). Also pluripotent (IFNγ+IL-2+) effector memory T cell (CD4+ CD62L- CD45RO+) responses, previously shown to be significantly increased by CPS immunization (8), were again induced (p=0.013), but did not differentiate between protected and unprotected volunteers (OR=1.6; 95% CI 0.5-4.9; p=0.41; data not shown).

CD107a expressing CD4 T cells presented as the clearest marker associated with protection, consistently higher in fully protected subjects from I1 onwards (**Figure 2.4A**), and independent of immunization dose (**Figure 2.4B**). A significant correlation was found between CD107a expression by CD4 T cells after one immunization and prepatent period after challenge-infection in all TS+ (Spearman's rho=0.69; p=0.013, **Figure 2.4C**). The proportion CD107a+ CD4 T cells in the control subject who developed parasitemia significantly later than the other controls (i.e. day 13.5 versus day 9-10.5), was at baseline on average 2.8 fold higher than in the other subjects. Possibly, the inherently higher response in this volunteer contributed to delayed pre-patency after challenge.

CD107a+ CD4 T cells expressed proportionally more granzyme B (7.4% versus 0.39% on C-1; p<0.0008) in protected subjects, indicative for their cytotoxic phenotype, and IFNγ (13.3% versus 0.39% on C-1; p<0.0001) than CD4 T cells negative for CD107a (**Figure 2.4D** and **Figure 2.4E**). CD8 T cells, traditionally considered the cytotoxic subclass of T cells, indeed contained a larger proportion of CD107a positive cells at baseline than CD4 T cells when unstimulated (uRBC); 0.39% versus 0.19% respectively; p<0.0001 (all volunteers). However, the proportion of *Pf*-specific degranulation of CD8 T cells was not notably increased by CPS immunization (p=0.44), in contrast to CD4 T cells (p<0.0001, **Figure 2.S2A&B**).

Both CD107a expression by CD4 T cells and granzyme B production by CD8 T cells remained significantly elevated up to twenty weeks after the challenge-infection (C+140) (p<0.05 and p<0.01; **Figure 2.5A** and **2.5B**), demonstrating longevity of the CPS-induced T cell response.

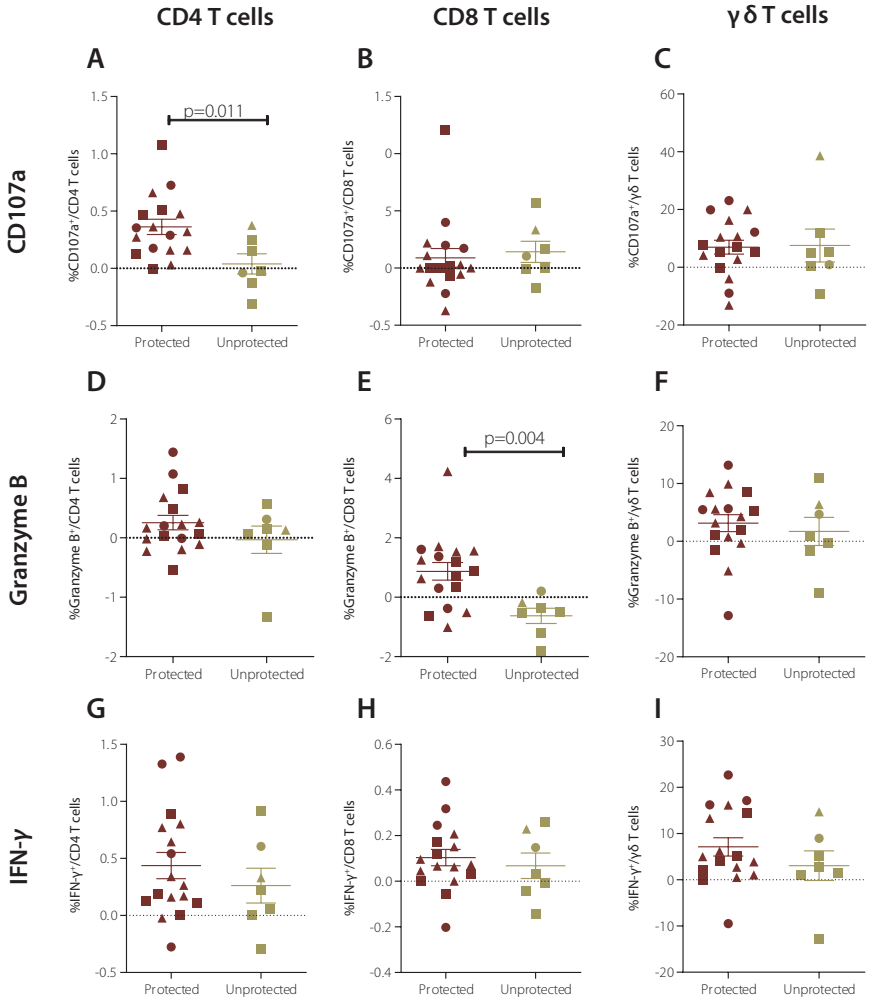

Figure 2.3 Cytotoxic immune responses upon in vitro PfRBC stimulation at one day before challenge infection (C-1).

*Each symbol represents a single protected (**red symbols**) or CPS-immunized unprotected (**brown symbols**) individual from group 1 (dots), group 2 (triangles) or group 3 (squares). Horizontal bars and whiskers represent means and SEMs. Panels show CD107a+ CD4 (**A**), CD8 (**B**) and γδ (**C**) T cells, granzyme B expression on CD4 (**D**), CD8 (**E**) and γδ (**F**) T cells and IFNγ+ CD4 (**G**), CD8 (**H**) and γδ (**I**) T cells. Values are corrected for uRBC background and for baseline-response before immunization. Background responses to uRBC stimulation were 0.19±0.01, 0.41±0.02 and 0.61±0.05 for CD107a, 1.65±0.50, 15.34±1.46 and 64.56±1.74 for granzyme B and 0.09±0.00, 0.07±0.00 and 0.14±0.01 for IFNγ, on CD4, CD8 and γδT cells respectively (mean ±SEM, calculated for all volunteers on both baseline and C-1). High uRBC granzyme B responses in CD8 and γδ T cells indicate that a significant percentage of these cells contains granzyme B even in a resting situation. uRBC responses did not change significantly from baseline for any of the readouts. The differences between responses of protected and unprotected volunteers in the graphs without a p-value are non-significant. The differences between protected and unprotected volunteers are calculated using logistic regression.*

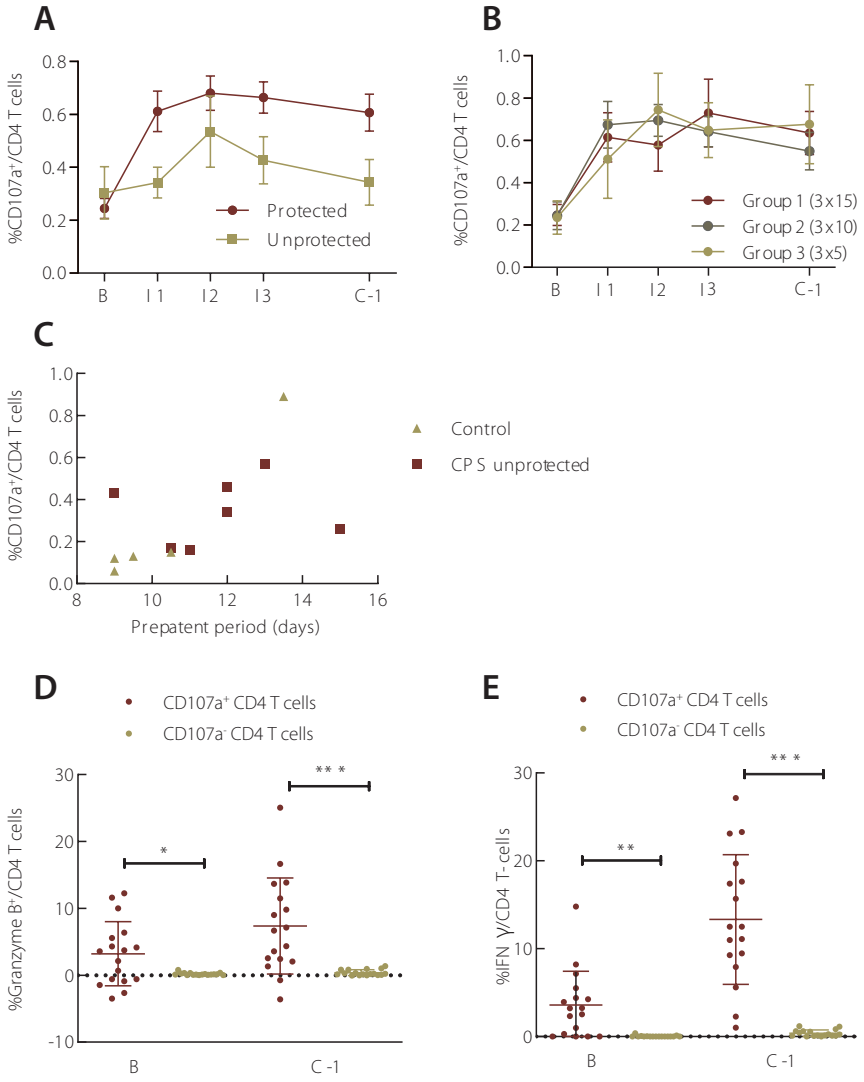

Figure 2.4 Cytotoxic profile of CPS-induced CD4 T cells.

*(**A+B**) Induction of Pf-specific CD107a positive CD4 T cells was determined (**A**) in protected and unprotected CPS-immunized subjects over the course of immunization and (**B**) in protected subjects separated for each immunization dose. Horizontal bars and whiskers represent mean responses and SEM. (**C**) The relationship between Pf-specific CD107a CD4 T cells on I1 and the prepatent period after challenge for all TS+ volunteers (CPS-immunized and controls). Within protected CPS-immunized subjects, (**D**) granzyme B and (**E**) IFNγ production by CD107a+ (**red dots**) and CD107a- (**brown dots**) CD4 T cells was analyzed at baseline (B on x-axis) and after CPS immunization (C-1) in all protected subjects. Horizontal bars show the mean response. All data were corrected for uRBC background for every volunteer at each time point. Abbreviations on the x-axis: B= baseline; I= 27 days after indicated immunization; C-1=one day before challenge. *=p<0.05 **p<0.01 ***p<0.001*

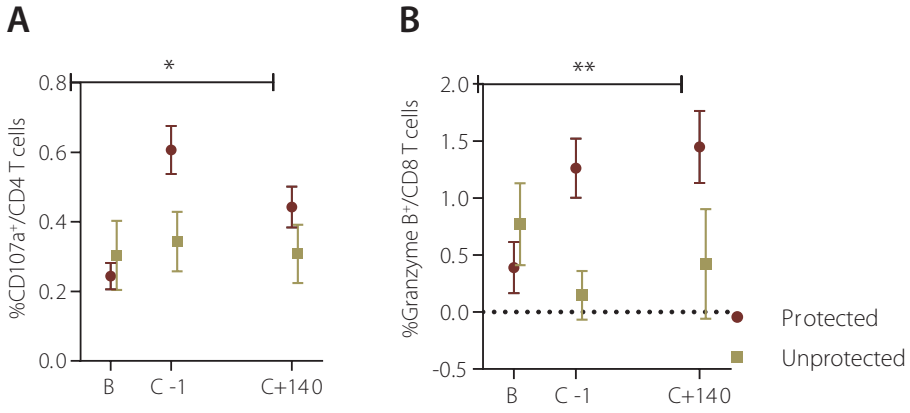

Figure 2.5 Longevity of cellular immune responses after CPS immunization.
*Pf-specific cellular immune responses (corrected for uRBC background) were assessed in protected (**red dots**) and unprotected (**brown squares**) CPS-immunized volunteers before CPS immunization (**B**), and before (C-1) and 20 weeks after challenge infection (C+140). Data are shown as mean ±SEM for (**A**) CD107a expression on CD4 T cells and (**B**) granzyme B production by CD8 T cells. Tests are performed separately for protected and immunized unprotected volunteers, by the repeated measures ANOVA (including all time points before and after immunizations) and the Dunnett's Multiple comparison post test, using B as control column. Only the test results of C+140 compared to baseline for protected volunteers are displayed. For immunized unprotected volunteers, all results were non-significant. *=p<0.05 **p<0.01*

DISCUSSION

We show that CPS immunization reproducibly and dose-dependently induces protection against a homologous challenge infection. With exposure to a total number of *Pf* infected mosquito bites as low as 30, CPS immunization still induces 89% protection in healthy volunteers. We furthermore demonstrate that markers of cytotoxic T cell responses are associated with protection against malaria after whole sporozoite immunization.

This study provides further support for the remarkable potency of the CPS-protocol to induce complete protection by using even lower numbers of *Pf*-infected mosquitoes than before (8). The observed dose-dependent protection is in line with results from RAS immunization trials with sporozoites administered either intravenously by needle and syringe (10) or by bites from irradiated infected mosquitoes (4). Although the delay of patency in unprotected CPS-immunized subjects was not statistically significant, the patterns of parasitemia indicate partial protection in some subjects. The unexpectedly delayed control subject hampered statistical significance but could be considered an outlier, possibly because of the inherently high baseline immune response. The establishment of a sub-optimal CPS immunization regimen inducing protection in 50% of the immunized volunteers with 3x5 mosquito bites will facilitate further studies of protective immune mechanisms against *Pf* malaria.

Our data provide evidence for a role of cytotoxic T cell responses in pre-erythrocytic immunity in humans. Due to obvious practical limitations, we only assessed immune cells in the peripheral blood, which may not necessarily reflect responses in the liver but rather represent a surrogate. The results of this exploratory analysis will have to be confirmed in future trials, and the functional relevance remains to be investigated.

'Classical' cytotoxic CD8 T cells can be activated by malaria antigen on infected hepatocytes via major histocompatibility complex (MHC) class I (25) and are associated with protection in a number of (animal) models (13, 14, 26). CD8 T cells are involved in protection in the murine CPS and RAS models (27-29), but their precise effector mechanisms remain subject of debate. They might either require direct contact with infected hepatocytes (13), or in fact be independent of granzyme B and/or other cytotoxic molecules, suggestive for a more indirect cytokine mediated effect by CD8 T cells (12) or other hepatic immune cells (30). In addition, a functional role for cytotoxic CD4 T cells is also conceivable as these cells can use cytolytic pathways such as granulysin, perforin and granzymes and FAS-L, as shown mostly in viral infections (31, 32). The protective role of CD4 T cells in murine malaria has been suggested, using *in vitro* experiments (33), and *in vivo* depletion (12) or passive transfer (34). Fur-

thermore, functional cytotoxic CD4 T cells, derived from RAS- or synthetic peptide immunized volunteers, are able to lyse autologous B cells pulsed with a peptide from the circumsporozoite protein (35-37). We used surface expression of CD107a (LAMP-1), a marker for cytotoxic degranulation, to phenotypically identify cytotoxic CD4 T cells (31). In order to directly kill a *Pf*- infected hepatocyte, parasite antigens should be presented in the context of MHC class II (MHCII) to the cytotoxic CD4 T cells. Although hepatocytes do not express MHCII in non-inflammatory circumstances, the presence of MHCII on human hepatocytes has been shown in a small number of patients with chronic hepatitis (38) and immune mediated liver disorders (39, 40). Functionally, over-expression of MHCII on hepatocytes in a transgenic mice model showed their capacity for co-stimulation, antigen-presentation and CD4 T cell activation (41). Only indirect evidence suggests that MHCII expression on mice hepatocytes may play a role in murine malaria (33, 42), and the presence of MHCII on hepatocytes in human malaria has never been studied. Here, we show for the first time that degranulating CD4 T cells are associated with protection in human malaria and already significantly induced after one immunization.

The observed lack of boosting by the second and third immunization may reflect a saturated response of antigen specific memory cells. This raises the possibility that fewer immunizations may be sufficient to induce protection, supported by the increased proportion of volunteers without parasitemia after the second and third immunization in Group 1 and 2. Moreover, the observed longevity of the immune response is in line with long-term protection after CPS immunization in a previous study (9).

The T_H1 cytokine IFNγ has been repeatedly shown to be an important effector molecule in protection against the malaria parasite (43), and the clear induction of T_H1 responses in our study corroborates earlier findings in both animals and humans after whole sporozoite immunization (8, 10, 12, 26, 27). We previously showed that a broad range of both innate and adaptive cellular subsets contribute to CPS-induced *Pf*-specific IFNγ production (16), which is sustained at least up to 2.5 years after immunization (9). IFNγ production alone, however, does not correlate with protection in neither RAS (10) nor our CPS model. Also production of both IFNγ and IL-2 by effector memory CD4 T cells, and IFNγ production by γδ-T cells, although clearly increased in immunized volunteers (8, 16), did not differentiate between protected and unprotected volunteers.

During CPS immunization, four protected subjects did not show parasitemia by qPCR at any measured time point, not even after the first immunization. A possible explanation is that the number of merozoites released from the liver is too low for qPCR detection. A strong primary innate immune response

may be responsible for clearing sporozoites and/or killing infected hepatocytes upon first encounter. Previous studies in mice indeed showed that inflammatory cytokines IL-1 and IL-6 block pre-erythrocytic development in mice (16, 44). Alternatively, chloroquine may have contributed to the decreased, i.e. undetectable number of parasites released from the liver either by direct killing, or indirectly by stimulating the immune system.

Antigen recognition and immune cell activation are essential for an effective response. To investigate pre-erythrocytic cellular immune responses, stimulation with cultured *Pf* liver stages would be preferred, but this is currently impossible. We therefore used asexual blood stage parasites for our experiments and although responses to purely pre-erythrocytic antigens may be missed, the majority of potential memory responses are likely detected upon *Pf*RBC stimulation, given the large overlap between liver and blood stage antigens (45). Future antigen screening by stimulation with a comprehensive library of pre-erythrocytic and cross-stage proteins or peptides, and subsequent functional studies focussing on cytotoxic T cells will further identify and delineate the specificity of protective responses (33, 46).

In conclusion, we identified two *in vitro* cellular cytotoxic immune markers that are associated with protection against malaria in a controlled clinical setting. Furthermore, this study confirms the robustness of CPS immunization as a highly efficient and reproducible immunization strategy for complete homologous protection. Further exploration of immune responses induced by CPS immunization will make important contributions to pre-erythrocytic malaria vaccine development and clinical testing.

ACKNOWLEDGMENTS

We thank all the trial volunteers for their participation in this study. We also thank K. Suijk-Benschop, J. Fehrmann-Naumann, C. Prins, E. Jonker, G. Hardeman and S. ten Velden-Schipper for blood collection and their good care for the volunteers. The LUMC department of Medical Microbiology for facilitating parasitological diagnosis and M. Erkens, T. Arens, J. van der Slot, H. Gerritsma, F. van de Sande, J. van Schie, E. Brienen, J. Schelfaut, J. Verweij, J.Kromhout, E. van Oorschot and M. Beljon for reading many thick smears. We thank C. Janse for his unlimited hospitality, M. Bootsma for her cardiac monitoring of the trial volunteers, W. Graumans and R. Siebelink-Stoter for culturing parasites and J. Klaassen, L. Pelser-Posthumus, J. Kuhnen, and A. Pouwelsen for generating infected mosquitoes and for assistance with immunizing and challenging the volunteers, G. Bastiaens and J. Wiersma for assistance with the immunizations and challenge and P. Houzé for the chloroquine measurements. We thank the members of the Safety Monitoring Committee, J.A. Romijn, M. de Boer and M. Laurens for their participation, guidance and safety recommendations throughout the trial. We also thank J. Verweij for the set-up and supervision of the DNA isolation and qPCR, and E. Brienen for performing these molecular tests, CromSource for clinical monitoring, the staff from the LUMC Central Clinical Biochemistry and Haematology Laboratories and the staff from the LUMC Pharmacy for making this study possible, A. Jansens, and D. Zimmerman for their administrative support, K. Teelen for help with sample logistics, M. Vos and B. van Schaijk for molecular analysis of the parasite batches and W. Nahrendorf for critically reading the manuscript.

The authors declare no conflict of interest. This work was supported by ZonMw [project number 95110086] and the Dioraphte foundation [project number 12010100]. The research leading to these results has furthermore received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement n°242095 (EviMalar). ACT was supported by an European Malaria Vaccine Development Association (EMVDA) fellowship.

REFERENCES

1. WHO. World malaria report 2013.
2. Crompton PD, Pierce SK, Miller LH. Advances and challenges in malaria vaccine development. *J Clin Invest*. 2010;120(12):4168-78.
3. RTS'S Clinical Trials Partnership, Agnandji ST, Lell B, Fernandes JF, Abossolo BP, Methogo BG, et al. A phase 3 trial of RTS,S/AS01 malaria vaccine in African infants. *N Engl J Med*. 2012;367(24):2284-95.
4. Hoffman SL, Goh LM, Luke TC, Schneider I, Le TP, Doolan DL, et al. Protection of humans against malaria by immunization with radiation-attenuated *Plasmodium falciparum* sporozoites. *J Infect Dis*. 2002;185(8):1155-64.
5. Sauerwein RW, Roestenberg M, Moorthy VS. Experimental human challenge infections can accelerate clinical malaria vaccine development. *Nat Rev Immunol*. 2011;11(1):57-64.
6. McCarthy JS, Good MF. Whole parasite blood stage malaria vaccines: a convergence of evidence. *Hum Vaccin*. 2010;6(1):114-23.
7. Butler NS, Vaughan AM, Harty JT, Kappe SH. Whole parasite vaccination approaches for prevention of malaria infection. *Trends Immunol*. 2012;33(5):247-54.
8. Roestenberg M, McCall M, Hopman J, Wiersma J, Luty AJ, van Gemert GJ, et al. Protection against a malaria challenge by sporozoite inoculation. *N Engl J Med*. 2009;361(5):468-77.
9. Roestenberg M, Teirlinck AC, McCall MBB, Teelen K, Makamdop KN, Wiersma J, et al. Long-term protection against malaria after experimental sporozoite inoculation: an open-label follow-up study. *Lancet*. 2011;377(9779):1770-6.
10. Seder RA, Chang LJ, Enama ME, Zephir KL, Sarwar UN, Gordon IJ, et al. Protection Against Malaria by Intravenous Immunization with a Nonreplicating Sporozoite Vaccine. *Science*. 2013;341(6152):1359-65.
11. Bijker EM, Bastiaens GJ, Teirlinck AC, van Gemert GJ, Graumans W, van de Vegte-Bolmer M, et al. Protection against malaria after immunization by chloroquine prophylaxis and sporozoites is mediated by preerythrocytic immunity. *Proc Natl Acad Sci U S A*. 2013;110(19):7862-7.
12. Doolan DL, Hoffman SL. The complexity of protective immunity against liver-stage malaria. *J Immunol*. 2000;165(3):1453-62.
13. Overstreet MG, Cockburn IA, Chen YC, Zavala F. Protective CD8 T cells against *Plasmodium* liver stages: immunobiology of an 'unnatural' immune response. *Immunol Rev*. 2008;225:272-83.
14. Tsuji M. A retrospective evaluation of the role of T cells in the development of malaria vaccine. *Exp Parasitol*. 2010;126(3):421-5.
15. Verhage DF, Telgt DS, Bousema JT, Hermsen CC, van Gemert GJ, van der Meer JW, et al. Clinical outcome of experimental human malaria induced by *Plasmodium falciparum*-infected mosquitoes. *Neth J Med*. 2005;63(2):52-8.
16. Teirlinck AC, McCall MBB, Roestenberg M, Scholzen A, Woestenenk R, de Mast Q, et al. Longevity and composition of cellular immune responses following experimental *Plasmodium falciparum* malaria infection in humans. *PLoS Pathog*. 2011;7(12):e1002389.

17. Firth D. Bias reduction of maximum likelihood estimates. *Biometrika*. 1993;80(1):27-38.

18. Heinze G. A comparative investigation of methods for logistic regression with separated or nearly separated data. *Statistics in medicine*. 2006;25(24):4216-26.

19. R Foundation for Statistical Computing. R: A language and environment for statistical computing. http://www.R-project.org/

20. Heinze G, Ploner M, Dunkler D, Southworth H. logistf: Firth's bias reduced logistic regression. R package version 1.21 http://CRAN.R-project.org/package=logistf

21. Harrell Jr FE. rms: Regression Modeling Strategies. R package version 4.1-3. http://CRAN.R-project.org/package=rms

22. Goeman JJ. L-1 Penalized Estimation in the Cox Proportional Hazards Model. *Biometrical Journal*. 2010;52(1):70-84.

23. Goeman JJ. Penalized R package, version 0.9-42.

24. Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez JC, et al. pROC: an open-source package for R and S+ to analyze and compare ROC curves. *BMC Bioinformatics*. 2011;12:77.

25. Ma J, Trop S, Baer S, Rakhmanaliev E, Arany Z, Dumoulin P, et al. Dynamics of the major histocompatibility complex class I processing and presentation pathway in the course of malaria parasite development in human hepatocytes: implications for vaccine development. *PLoS One*. 2013;8(9):e75321.

26. Epstein JE, Tewari K, Lyke KE, Sim BK, Billingsley PF, Laurens MB, et al. Live attenuated malaria vaccine designed to protect through hepatic CD8(+) T cell immunity. *Science*. 2011;334(6055):475-80.

27. Belnoue E, Costa FT, Frankenberg T, Vigario AM, Voza T, Leroy N, et al. Protective T cell immunity against malaria liver stage after vaccination with live sporozoites under chloroquine treatment. *J Immunol*. 2004;172(4):2487-95.

28. Nganou-Makamdop K, van Gemert GJ, Arens T, Hermsen CC, Sauerwein RW. Long term protection after immunization with *Plasmodium berghei* sporozoites correlates with sustained IFNgamma responses of hepatic CD8+ memory T cells. *PLoS One*. 2012;7(5):e36508.

29. Cockburn IA, Amino R, Kelemen RK, Kuo SC, Tse SW, Radtke A, et al. In vivo imaging of CD8+ T cell-mediated elimination of malaria liver stages. *Proc Natl Acad Sci U S A*. 2013;110(22):9090-5.

30. Kimura K, Kimura D, Matsushima Y, Miyakoda M, Honma K, Yuda M, et al. CD8+ T cells specific for a malaria cytoplasmic antigen form clusters around infected hepatocytes and are protective at the liver stage of infection. *Infect Immun*. 2013;81(10):3825-34.

31. Marshall NB, Swain SL. Cytotoxic CD4 T cells in antiviral immunity. *BioMed Research International*. 2011;2011:Article ID 954602.

32. Brown DM. Cytolytic CD4 cells: Direct mediators in infectious disease and malignancy. *Cell Immunol*. 2010;262(2):89-95.

33. Renia L, Marussig MS, Grillot D, Pied S, Corradin G, Miltgen F, et al. In vitro activity of CD4+ and CD8+ T lymphocytes from mice immunized with a synthetic malaria peptide. *Proc Natl Acad Sci U S A*. 1991;88(18):7963-7.

34. Tsuji M, Romero P, Nussenzweig RS, Zavala F. CD4+ cytolytic T cell clone confers protection against murine malaria. *J Exp Med*. 1990;172(5):1353-7.

35. Moreno A, Clavijo P, Edelman R, Davis J, Sztein M, Herrington D, et al. Cytotoxic Cd4+ T-Cells from a Sporozoite-Immunized Volunteer Recognize the *Plasmodium falciparum* Cs Protein. *International Immunology*. 1991;3(10):997-1003.

36. Frevert U, Nardin E. Cellular effector mechanisms against *Plasmodium* liver stages. *Cell Microbiol*. 2008;10(10):1956-67.

37. Frevert U, Moreno A, Calvo-Calle JM, Klotz C, Nardin E. Imaging effector functions of human cytotoxic CD4+ T cells specific for *Plasmodium falciparum* circumsporozoite protein. *Int J Parasitol*. 2009;39(1):119-32.

38. Franco A, Barnaba V, Natali P, Balsano C, Musca A, Balsano F. Expression of class I and class II major histocompatibility complex antigens on human hepatocytes. *Hepatology*. 1988;8(3):449-54.

39. Senaldi G, Lobo-Yeo A, Mowat AP, Mieli-Vergani G, Vergani D. Class I and class II major histocompatibility complex antigens on hepatocytes: importance of the method of detection and expression in histologically normal and diseased livers. *J Clin Pathol*. 1991;44(2):107-14.

40. Lobo-Yeo A, Senaldi G, Portmann B, Mowat AP, Mieli-Vergani G, Vergani D. Class I and class II major histocompatibility complex antigen expression on hepatocytes: a study in children with liver disease. *Hepatology*. 1990;12(2):224-32.

41. Herkel J, Jagemann B, Wiegard C, Lazaro JF, Lueth S, Kanzler S, et al. MHC class II-expressing hepatocytes function as antigen-presenting cells and activate specific CD4 T lymphocytes. *Hepatology*. 2003;37(5):1079-85.

42. Renia L, Grillot D, Marussig M, Corradin G, Miltgen F, Lambert PH, et al. Effector functions of circumsporozoite peptide-primed CD4+ T cell clones against Plasmodium yoelii liver stages. *J Immunol*. 1993;150(4):1471-8.

43. McCall MB, Sauerwein RW. Interferon-gamma--central mediator of protective immune responses against the pre-erythrocytic and blood stage of malaria. *J Leukoc Biol*. 2010;88(6):1131-43.

44. Vreden SG, van den Broek MF, Oettinger MC, Verhave JP, Meuwissen JH, Sauerwein RW. Cytokines inhibit the development of liver schizonts of the malaria parasite *Plasmodium berghei* in vivo. *Eur J Immunol*. 1992;22(9):2271-5.

45. Tarun AS, Peng X, Dumpit RF, Ogata Y, Silva-Rivera H, Camargo N, et al. A combined transcriptome and proteome survey of malaria parasite liver stages. *Proc Natl Acad Sci U S A*. 2008;105(1):305-10.

46. Doolan DL, Southwood S, Freilich DA, Sidney J, Graber NL, Shatney L, et al. Identification of *Plasmodium falciparum* antigens by antigenic analysis of genomic and proteomic data. *Proc Natl Acad Sci U S A*. 2003;100(17):9952-7.

47. Adegnika AA, Verweij JJ, Agnandji ST, Chai SK, Breitling LP, Ramharter M, et al. Microscopic and sub-microscopic *Plasmodium falciparum* infection, but not inflammation caused by infection, is associated with low birth weight. *Am J Trop Med Hyg*. 2006;75(5):798-803.

48. Lejeune D, Souletie I, Houze S, Le bricon T, Le bras J, Gourmel B, et al. Simultaneous determination of monodesethylchloroquine, chloroquine, cycloguanil and proguanil on dried blood spots by reverse-phase liquid chromatography. *J Pharm Biomed Anal*. 2007;43(3):1106-15.

SUPPLEMENTARY INFORMATION

CPS immunization and challenge

All subjects received a standard prophylactic regimen of chloroquine consisting of a loading dose of 300 mg on each of the first two days and then 300 mg once a week for a total duration of 14 weeks. During this period, all subjects were exposed three times to the bites of *Anopheles stephensi* mosquitoes at monthly intervals starting eight days after the first chloroquine dose as described previously (11). All volunteers were exposed to bites from exactly 15 mosquitoes at each session, but the number of NF54 *Pf* infected versus uninfected mosquitoes varied per group: five subjects received three times bites from 15 infected mosquitoes (Group 1), ten subjects received three times bites from 10 infected and 5 uninfected mosquitoes (Group 2), ten subjects received three times bites from 5 infected and 10 uninfected mosquitoes (Group 3) and five control subjects received three times bites from 15 uninfected mosquitoes (Group 4). From day 6 to 10 after each immunization, subjects were checked daily on an outpatient basis and blood was drawn for peripheral blood smears, standard haematological measurements and cardiovascular safety markers and stored for retrospective analysis of parasitemia by quantitative real-time PCR (qPCR) (47).

After the challenge-infection, volunteers were checked twice daily on an outpatient basis from day 5-21 for (un)solicited symptoms and signs. As soon as parasites were detected by thick smear, subjects were treated with a standard curative regimen of 1000 mg atovaquone and 400 mg proguanil once daily for three days, according to Dutch national guidelines. If subjects remained thick smear negative, they were presumptively treated with the same curative regimen on day 21 after challenge infection. Chloroquine levels one day before challenge were measured in EDTA-plasma by liquid chromatography and were below detection limit (5 µg/L) in all volunteers one day before challenge (48).

Retrospectively, parasitemia was quantified on day six until day ten after each immunization and from day five until day 21 after challenge by qPCR using *Pf* standard curves prepared by DNA extraction from titrated samples of ring-infected cells (47). Adverse events (AEs) were recorded as described previously (11).

Platelet counts were determined in EDTA-anticoagulated blood with the Sysmex XE-2100 (Sysmex Europe GmbH, Norderstedt, Germany). D-dimer concentrations were assessed in citrate plasma by STA-R Evolution (Roche Diagnostics, Almere, The Netherlands).

PBMC isolation and cryopreservation

Venous whole blood was collected into citrated vacutainer cell preparation tubes (CPT; Becton and Dickinson) and stored at room temperature for a maximum of 4 hours; PBMCs were isolated by centrifugation and washed four times in ice-cold phosphate-buffered saline (PBS). Cells were counted and cryopreserved at a concentration of 10^7 cells/ml in ice-cold foetal-calf serum (Gibco) containing 10% dimethylsulfoxide (Merck, Germany) using Mr. Frosty freezing containers (Nalgene). Samples were stored in vapour-phase nitrogen.

In vitro *Pf*- infected erythrocyte re-stimulation assay

PBMC were thawed, washed twice in Dutch-modified RPMI 1640 (Gibco/ Invitrogen) and counted in 1% trypan blue containing 5% zap-oglobin II Lytic Reagent (Beckman Coulter) using a Neubauer improved bright line counting chamber (Marienfield, Germany); median cell recovery was 80%. PBMCs were *in vitro* re-stimulated with cryopreserved NF54 *Pf*-infected erythrocytes (*Pf*RBC) as described previously (16). Cells were re-suspended in complete culture medium (Dutch-modified RPMI 1640 containing 2 mM glutamine, 1mM pyruvate, 0.05 mM gentamycine and 10% human A+ serum, (Sanquin, Nijmegen) at a final concentration of $2.5x10^6$/ml. PBMC were transferred into polystyrene 96-well round-bottom plates and stimulated in duplicate wells with either $5x10^6$/ml (final concentration) cryopreserved *Pf*RBC or uRBC (uninfected erythrocytes) in a total volume of 110 µl/well for 24 hours at 37°C/ 5%CO_2. For the last four hours, 10 µg/ml Brefeldin A (Sigma-Aldrich) and 2µM monensin (eBioscience) were added, based on pilot experiments. In positive control wells, PMA (50 ng/ml, Sigma-Aldrich) and ionomycin (1 mg/ml, Sigma-Aldrich) were added the last four hours. After a total of 24 hours, cells were harvested and stained.

Flow cytometry analysis

PBMCs were co-incubated during the 24 hour-stimulation with CD107a Pacific Blue (Biolegend, clone H4A3). All cells were transferred to a polystyrene V-bottom plate and washed twice with 200µl PBS. Next, cells were stained with Live/Dead fixable dead cell stain dye aqua (Invitrogen) in 50 µl PBS for 30 minutes at 4°C. After washing with PBS containing 0.5% bovine serum albumin (BSA, Sigma-Aldrich) cells were stained with antibodies against the surface markers CD3 PerCP (Biolegend, clone UCHT1), CD4 ECD (Beckman-Coulter, clone SF-

CI12T4D11) CD8 APC-H7 (BD Biosciences, clone SK1), γδ-T cell receptor PE (Beckman-Coulter, clone IMMU510) and CD56 APC (eBioscience, clone MEM188) in 50 µl PBS containing 0.5% BSA for 30 minutes at 4°C. Cells were washed again and fixed in Foxp3 fixation/permeabilization buffer (eBioscience). Following a wash step with Foxp3 permeabilization buffer (eBioscience), cells were stained in permeabilization buffer containing granzyme B FITC (Biolegend, clone GB11) and IFNγ PeCy7 (Biolegend, clone 4S.B3). Cells were washed again in permeabilization buffer and kept cold and dark in fixation buffer (1% paraformaldehyde in PBS) until measured by flow cytometry on the same day. For every individual volunteer, all time points were thawed, stimulated and stained within the same experimental round. In a separate experiment, cells from the time points B and C-1 were *in vitro* re-exposed to *Pf* infected erythrocytes and stained for viability, γδ-T cell receptor PE, CD56 PE, CD3 PerCP, CD45RO ECD (Beckman-Coulter, clone mIgG2a), CD62L PeCy7 (Biolegend, clone DREG-56) CD4 Pacific Blue (eBioscience, clone OKT-4) CD8 AF700 (Biolegend, clone HIT8A), IFNγ FITC and IL-2 APC (eBioscience, clone MQ1-17H12) using the same protocol as described for the other staining panel.

Samples were acquired using a 9-color Cyan ADP (Beckman Coulter), each round using single stained cells for compensation. Per sample, a median of $93.8x10^3$ (range $12.5x10^3$ - $221x10^3$) singlet living lymphocytes were acquired. Data analysis was performed using FlowJo software (version 9.6; Tree Star). A representative example showing the gating strategy is shown in S3. The definition of cell positivity (for cytokines and cytotoxic molecules) was performed automatically, based on the MFI of unresponding PBMCs for each sample separately. Responses to uRBC were subtracted from the response to *Pf*RBC for every volunteer on every time point.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 5 unless mentioned otherwise. Differences between immunized unprotected and control volunteers in prepatent periods by thick smear and qPCR were tested by Mann-Whitney U test. Induction of cytotoxic immune responses on the time points I1, I2, I3 and C-1 were tested by the repeated measures ANOVA and the Dunnett's Multiple comparison post test, with baseline as control column. Induction of immune responses on 140 days after challenge (C+140) was tested separately for protected and immunized unprotected volunteers, by the repeated measures ANOVA (including all previous time points mentioned above) and the Dunnett's Multiple comparison post test, with baseline as control column.

The correlation of CD107a expression by CD4 T cells with the prepatent period, and the correlation of cellular immune responses with cumulative parasitemia during CPS immunization were assessed by non-parametric Spearman correlation. The proportion of CD107a+ CD4 vs CD8 T cells and the production of granzyme B and IFNγ on CD107a^{+} vs CD107a- CD4 T cells were tested by the paired Student's t-test. For the correlation of CD107a CD4 T cells with prepatent period after challenge, immune re-call responses to *Pf*RBC (corrected for uRBC stimulation background) were tested on the different time points, while for all other tests we assessed the change from baseline (B).

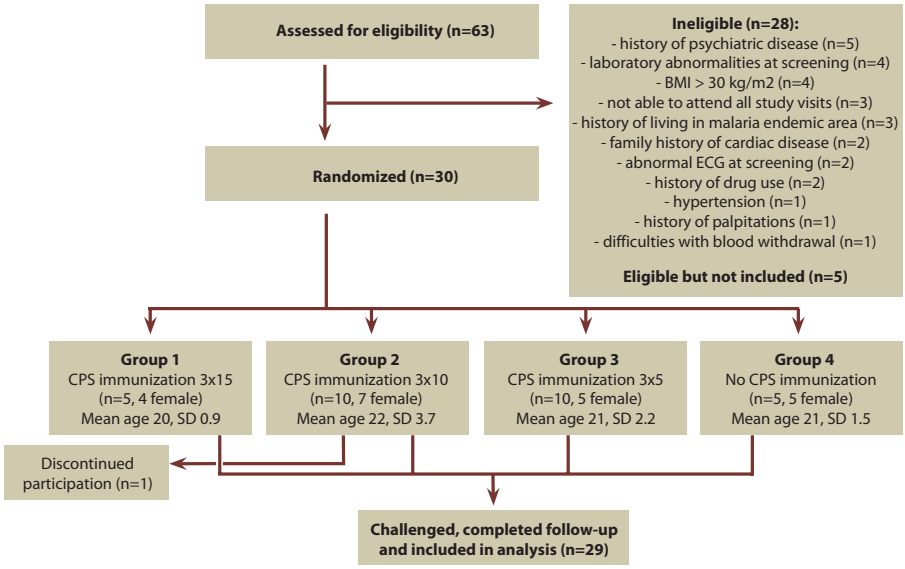

Figure 2.S1 Study flow diagram.
Twenty-five subjects were randomly assigned to receive different doses of CPS immunization in a double-blind fashion; five control subjects received bites from uninfected mosquitoes. One subject withdrew informed consent after the first immunization for reasons unrelated to the trial. Twenty-nine subjects received a challenge infection by the bites of five infected mosquitoes fifteen weeks after discontinuation of chloroquine chemoprophylaxis.

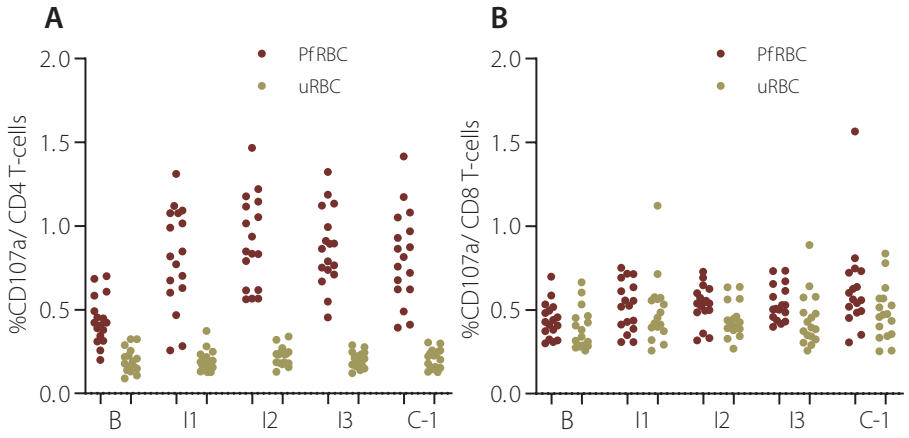

Figure 2.S2 Induction of cytotoxic CD4 and CD8 T cell responses by CPS immunization. *CD107a expression was assessed on (**A**) CD4 T cells and (**B**) CD8 T cells after stimulation with PfRBC (**red dots**) and uRBC (**brown dots**) before, during and after CPS immunization (protected subjects only). B= baseline; I= 27 days after indicated immunization; C-1=one day before challenge.*

Funding

This work was supported by ZonMw [project number 95110086] and the Dioraphte foundation [project number 12010100]. The research leading to these results has furthermore received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement n°242095 (EviMalar). ACT was supported by an European Malaria Vaccine Development Association (EMVDA) fellowship.

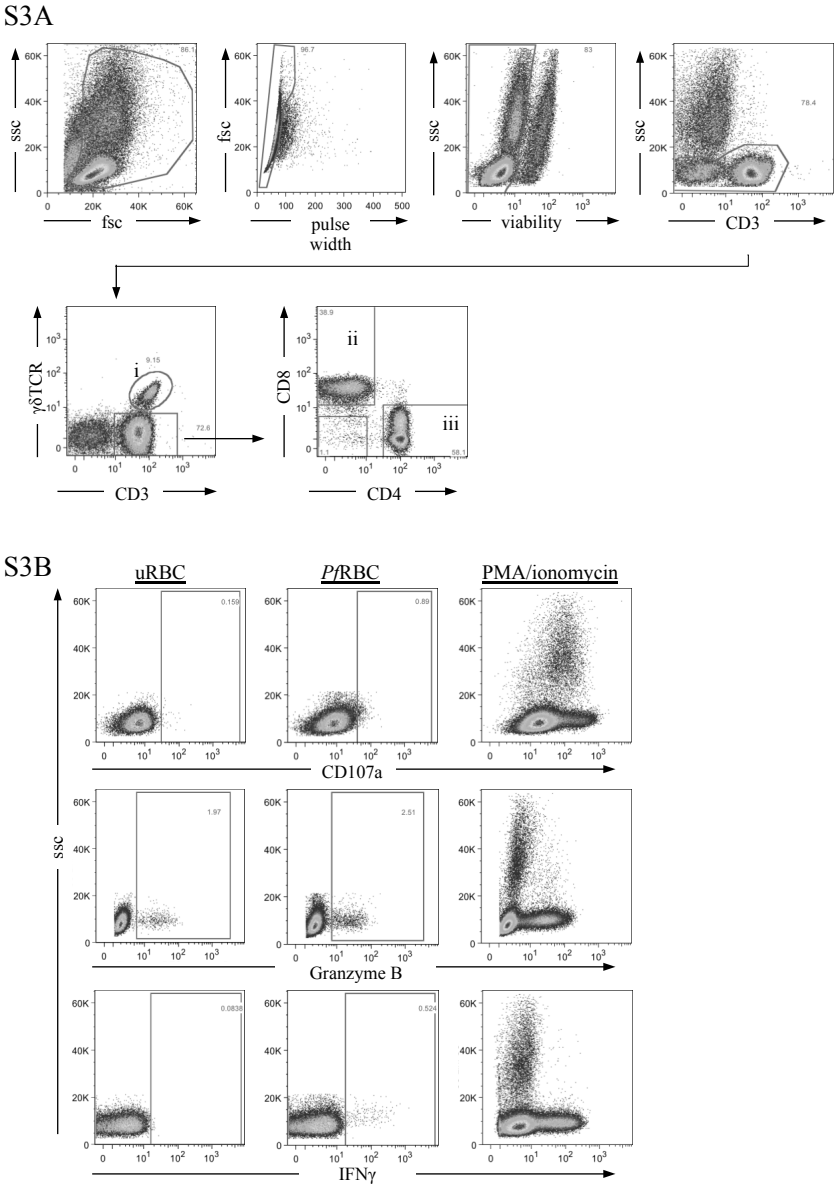

Figure S2.3 Flow cytometry gating strategy.
*(**A**) Representative flow cytometry plots for a uRBC stimulated sample from one volunteer at baseline (before immunization). Singlet viable CD3+ PBMC were subdivided into (i) γδT cells, (ii) CD8 T cells and (iii) CD4 T cells; No additional dump channel for CD14, CD19 and CD20 was used. (**B**) Gating of CD107a, granzyme B and IFNγ positive cells for uRBC, PfRBC and PMA/ionomycin re-stimulated cells at baseline. For uRBC and PfRBC stimulation CD4 T cells are shown, for PMA/ionomycin total viable PBMCs. Within each sample, gating of marker positive cells was performed automatically, based on the MFI of marker negative cells.*