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 SFCI12T4D11) 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 **Figure 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-

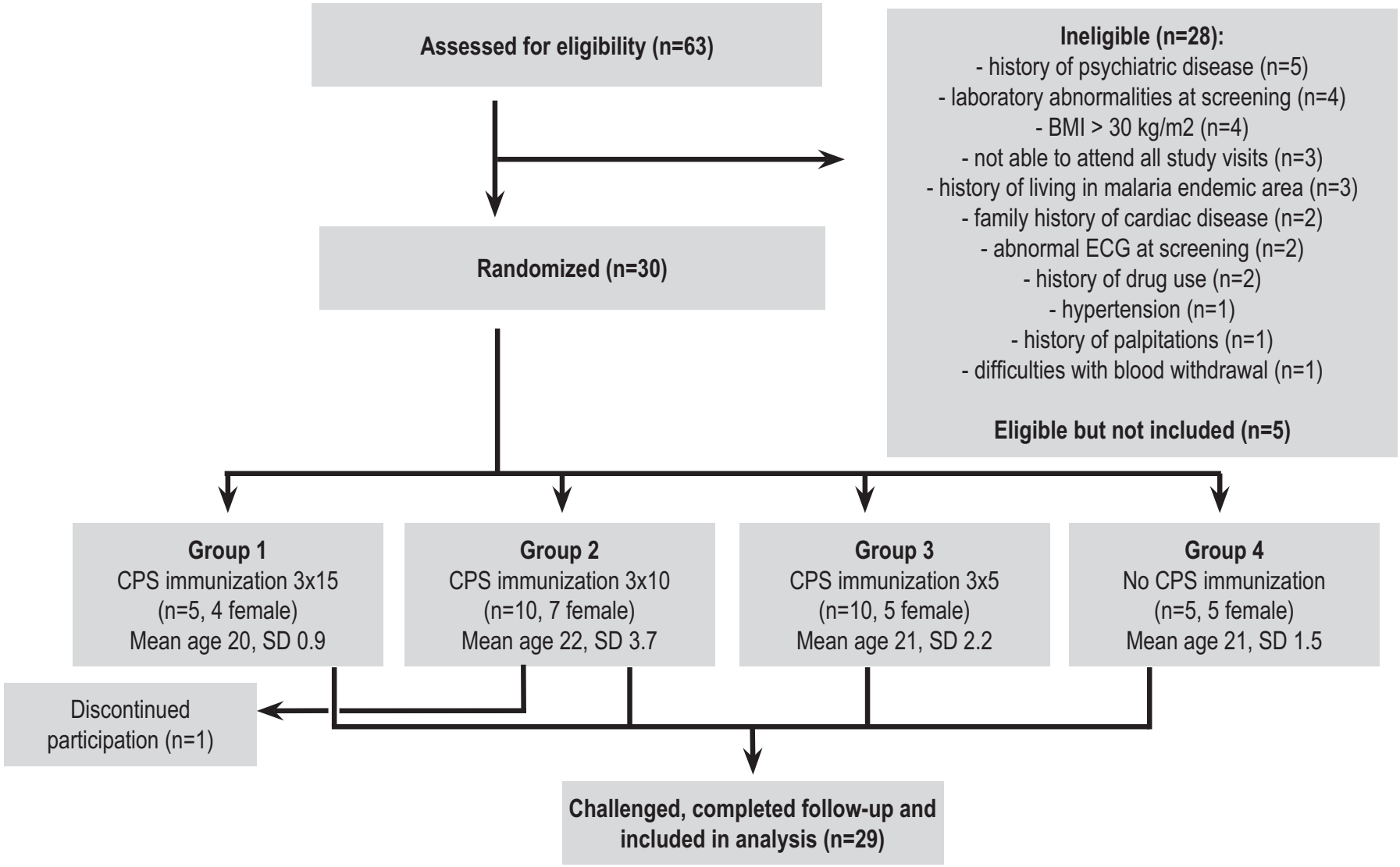
CD4 T cells were tested by the paired Student's t-test. For the correlation of CD107a CD4 T cells with prepatent period after challenge, immune re-call responses to *Pf*RBC (corrected for uRBC stimulation background) were tested on the different time points, while for all other tests we assessed the change from baseline (B).

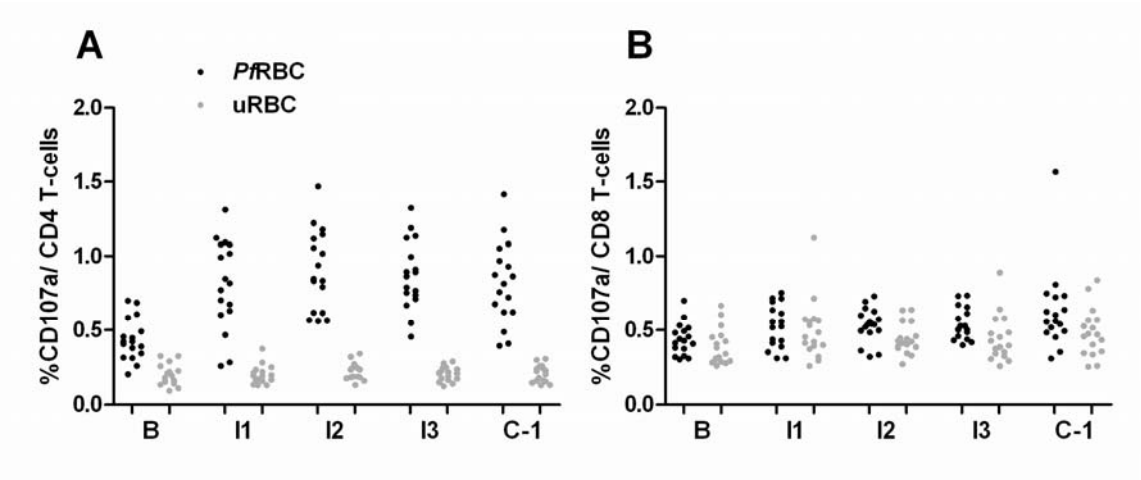
Supplementary Figure Legends

Figure 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 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 *Pf*RBC (black dots) and uRBC (grey dots) before, during and after CPS immunization (protected subjects only). B= baseline; I= 27 days after indicated immunization; C-1=one day before challenge.

Figure S3. 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, *Pf*RBC and PMA/ionomycin re-stimulated cells at baseline. For uRBC and *Pf*RBC 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.


Assessed for eligibility (n=63)
Ineligible (n=28):
- history of psychiatric disease (n=5)
- laboratory abnormalities at screening (n=4)
- BMI > 30 kg/m2 (n=4)
- not able to attend all study visits (n=3)
- history of living in malaria endemic area (n=3)
- family history of cardiac disease (n=2)
- abnormal ECG at screening (n=2)
- history of drug use (n=2)
- hypertension (n=1)
- history of palpitations (n=1)
- difficulties with blood withdrawal (n=1)
Eligible but not included (n=5)
Randomized (n=30)
Group 1
CPS immunization 3x15
(n=5, 4 female)
Mean age 20, SD 0.9
Group 2
CPS immunization 3x10
(n=10, 7 female)
Mean age 22, SD 3.7
Group 3
CPS immunization 3x5
(n=10, 5 female)
Mean age 21, SD 2.2
Group 4
No CPS immunization
(n=5, 5 female)
Mean age 21, SD 1.5
Discontinued participation (n=1)
Challenged, completed follow-up and included in analysis (n=29)


A
PfRBC
uRBC
%CD107a/ CD4 T-cells
2.0
1.5
1.0
0.5
0.0
B
I1
I2
I3
C-1
B
%CD107a/ CD8 T-cells
2.0
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B
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I2
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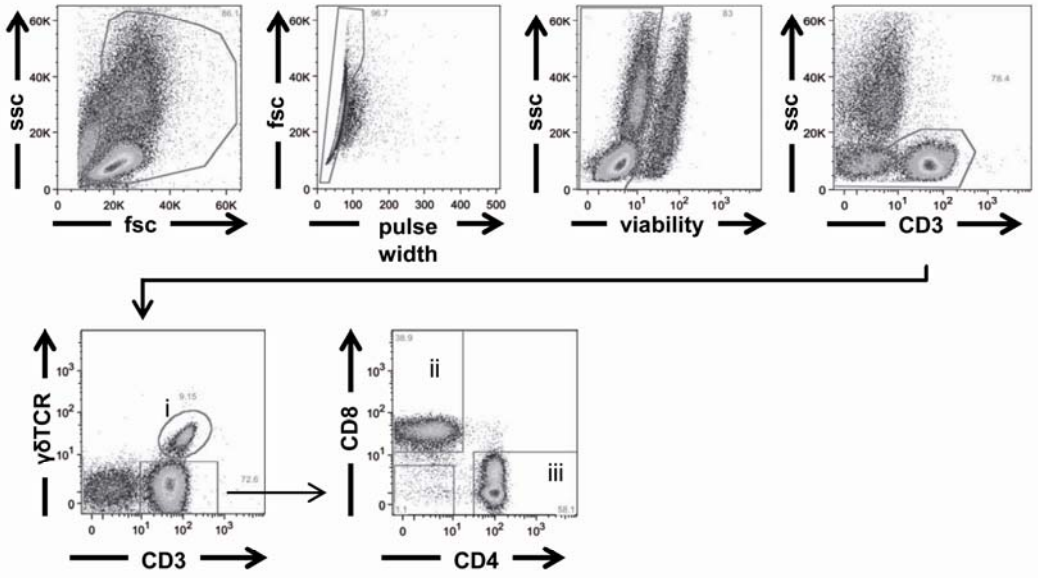
C-1

S3A


ssc
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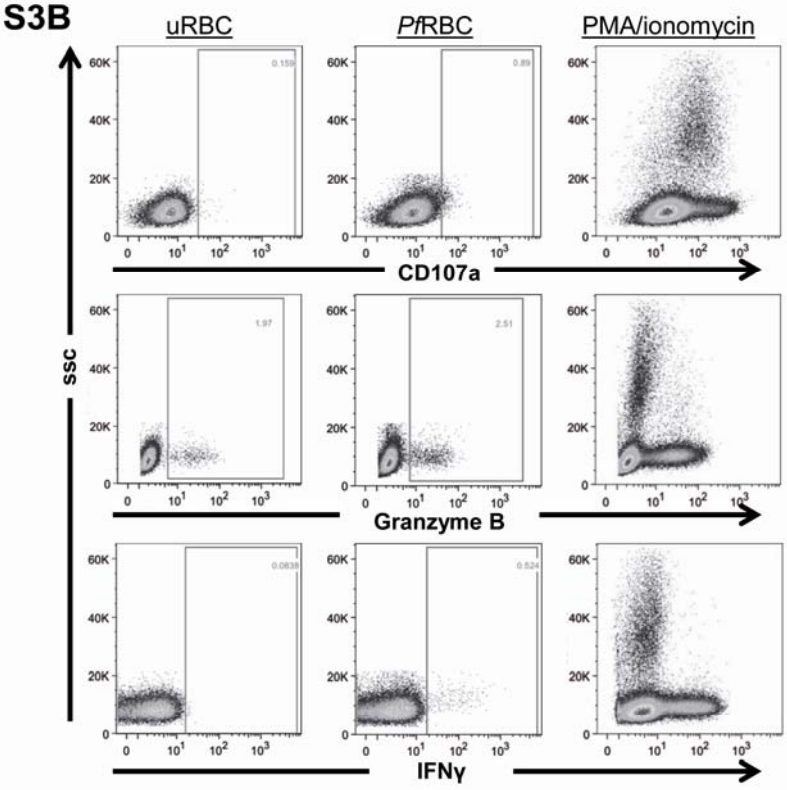
uRBC
PfRBC
PMA/ionomycin
CD107a
ssc
Granzyme B
IFNγ