

Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Roestenberg M, McCall M, Hopman J, et al. Protection against a malaria challenge by sporozoite inoculation. *N Engl J Med* 2009;361:468-77.

Supplementary methods

Serology

To assess antibody titers, asexual ELISAs were performed as previously described¹. CSP ELISAs were performed with (NANP)₆ NA (kind gift from G. Corradin) according to published methods². AMA-1 and GLURP ELISAs were performed according to a standard in-house protocol^{2,3}. In all ELISAs a plasma pool of 8 healthy malaria-naive Dutch volunteers was used as a negative control to define sample positivity (above mean+2SD of negative control). A plasma pool of Tanzanian adults (n=100) living in a highly malaria endemic area was used as reference positive control, defined to contain 100 arbitrary units (AU).

Immunofluorescence assays were performed by incubating plasma at 1/320 dilution with air-dried whole sporozoites or at 1/40 dilution with asynchronous cultures of NF54 strain asexual *P. falciparum* parasites, as previously described³.

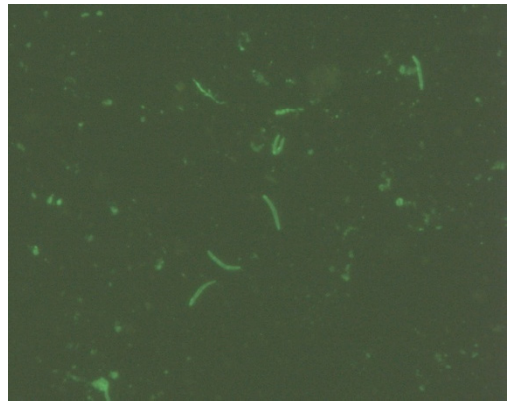
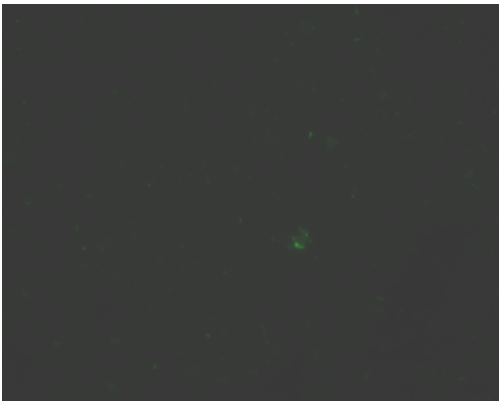
Cellular immunology

Cellular immune responses were assessed by *in vitro* stimulation assays. For use in these assays, Percoll-purified asynchronous asexual-stage cultures of NF54 strain parasites of 80-90% parasitemia, consisting of more than 95% schizonts/mature trophozoites, were washed, aliquoted and cryopreserved in advance. Mock-cultured uninfected erythrocytes (uRBC) were obtained similarly and served as control. Cryopreserved PBMCs were thawed immediately prior to use in *in vitro* stimulation assays, washed and resuspended in RPMI 1640 culture medium containing 2mM glutamine, 1mM pyruvate, 50µg/mL gentamycin and 10% pooled human AB⁺ serum (Sanquin, Nijmegen, NL), for a final concentration of 2.5x10⁶/mL. PBMCs were transferred into 96-well round-bottom plates (5x10⁵/well) and stimuli were added to duplicate wells. Stimuli included cryopreserved PfrBC (5x10⁶/mL), uRBC, PHA (10 µg/mL) or RPMI only. PBMCs were stimulated for 24 hours at 37°C/5%CO₂. 4 hours prior to cell harvest, 100µL/well supernatant was collected and replaced with 100 µL/well fresh culture medium containing 20 µg/mL brefeldin A (Sigma). PBMCs from both time points per volunteer were tested simultaneously and cells from two vaccinees and one control were always tested together.

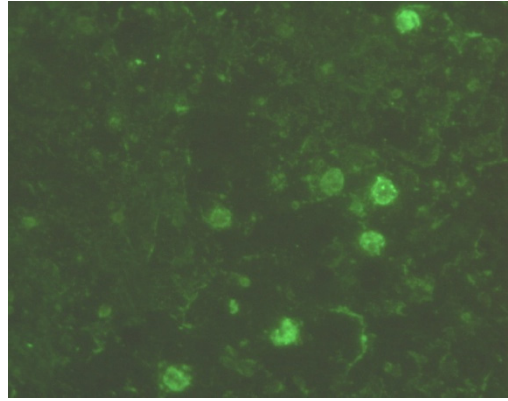
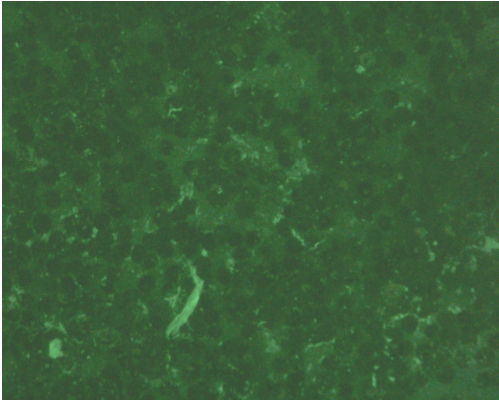
Following 24 hour *in vitro* stimulation, PBMCs were harvested, washed once in FACS buffer (0.5% BSA/PBS) and incubated for 15' with fluorescent mAbs against cell surface markers. Cells were washed again and incubated for 15' in fixation medium A (Caltag Laboratories, Carlsbad, CA) according to the manufacturer's instructions, washed again and incubated for 15' with fluorescent mAbs against intracellular cytokines in permeabilization medium B. After a final wash step, cells were resuspended in FACS buffer and read on a FACScalibur flowcytometer. The following fluorescent mAbs were used: CD3-PerCP, CD4-PerCP, CD8-PE (BD, Breda, NL), IFNγ-FITC, TNFα-PE, IL-2-APC, CD45RO-PE, CD62L-PeCy7, mouse IgG1-FITC & IgG2a-PE and rat IgG2a-APC isotype controls (all Ebioscience, Uithoorn, NL).

1. Bousema JT, Roeffen W, van der KM et al. Rapid onset of transmission-reducing antibodies in javanese migrants exposed to malaria in papua, indonesia. *Am J Trop Med Hyg* 2006; 74:425-31.
2. Remarque EJ, Faber BW, Kocken CH, Thomas AW. A diversity-covering approach to immunisation with Plasmodium falciparum AMA1 induces broader allelic recognition and growth inhibition responses in rabbits. *Infect Immun* 2008.
3. Hermsen CC, Verhage DF, Telgt DS et al. Glutamate-rich protein (GLURP) induces antibodies that inhibit in vitro growth of Plasmodium falciparum in a phase 1 malaria vaccine trial. *Vaccine* 2007; 25:2930-40.

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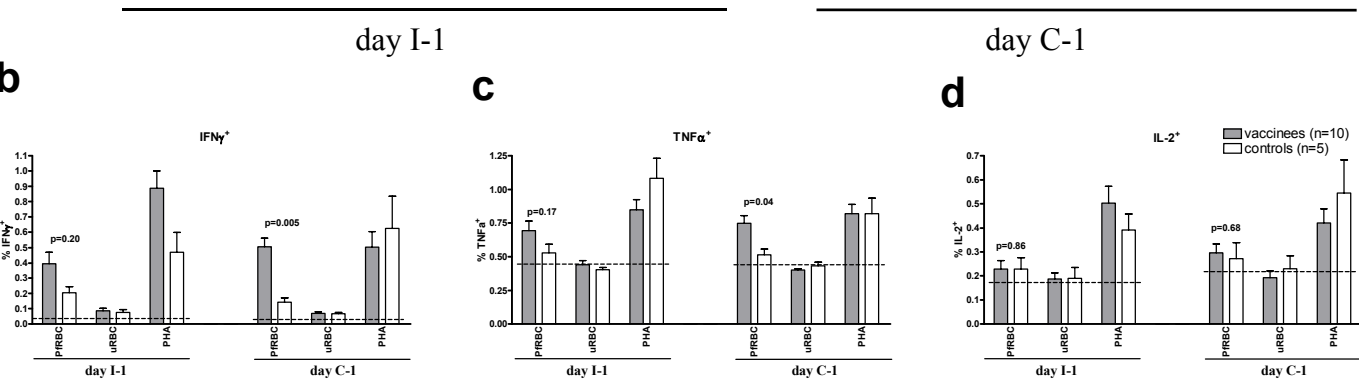
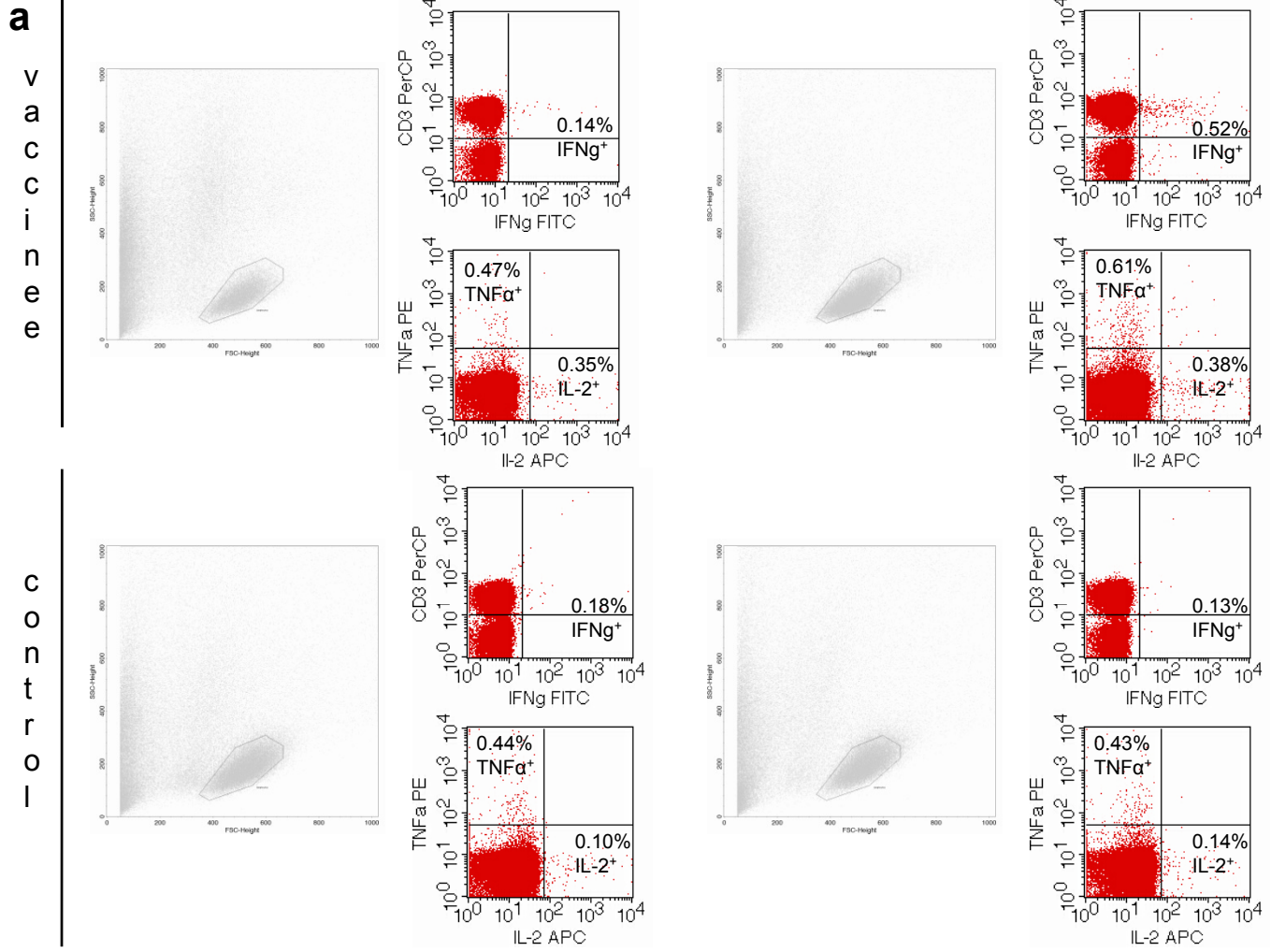


day I-1

day C-1

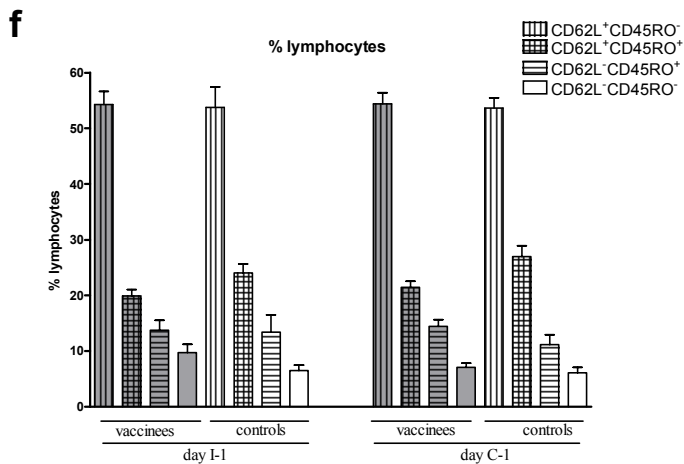
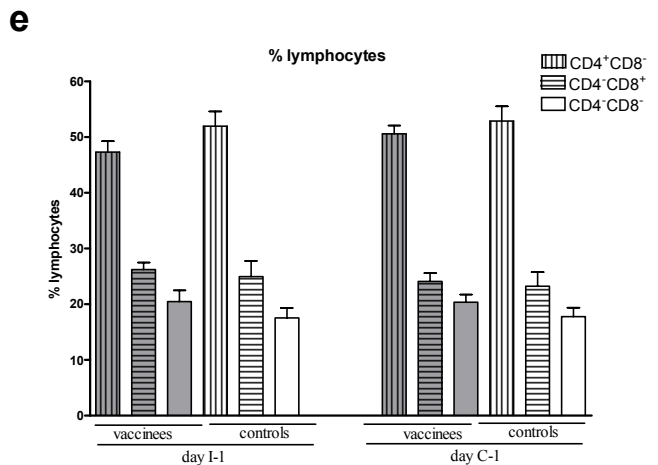
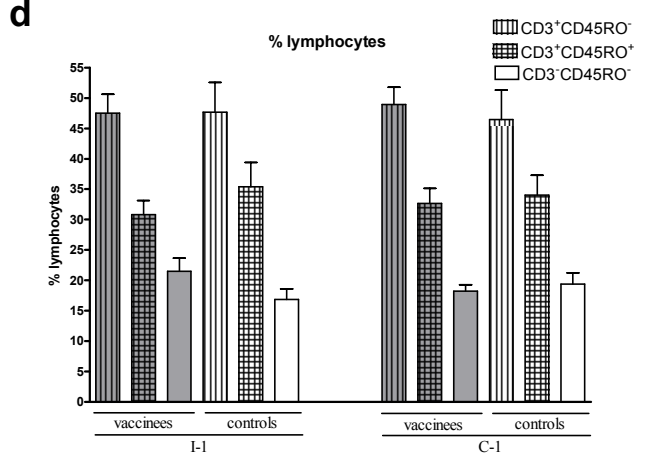
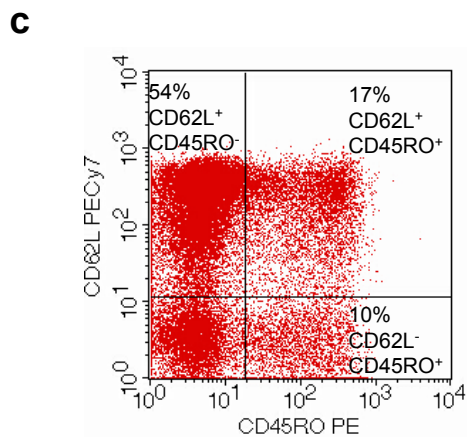
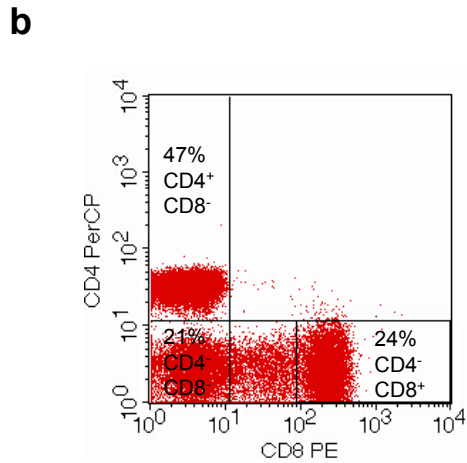
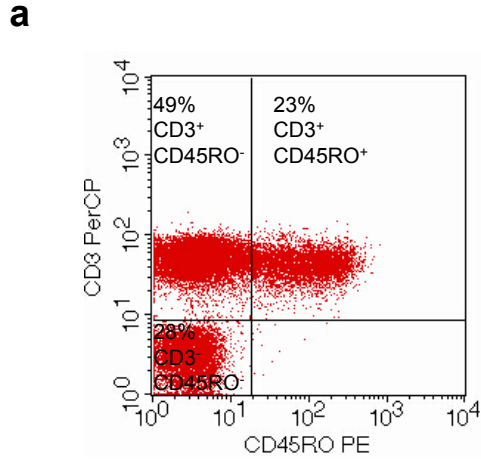
Supplementary figure 1: Representative immunofluorescence assay (IFA) images.

Plasma from a vaccinee collected prior to immunization (day I-1, left) or prior to challenge (day C-1, right), was incubated at 1/320 dilution with whole sporozoites (above) or at 1/40 dilution with an asynchronous culture of asexual blood-stage parasites (below).



Supplementary figure 2: *In vitro* cytokine responses to *P. falciparum* parasites by intracellular flow-cytometry.

a. Representative flow-cytometry plots of cytokine production by PBMC isolated prior to immunization (day I-1, left) or prior to challenge (day C-1, right) from a vaccinee (above) and a control volunteer (below) and stimulated *in vitro* for 24 hours with PfrBC. **b-d:** Percentage of vaccinee (shaded grey) and control (unshaded) lymphocytes collected prior to immunization (day I-1) and prior to challenge (day C-1) producing IFN γ (**b**), TNF α (**c**) and IL-2 (**d**), respectively, following *in vitro* stimulation with PfrBC or controls. Dotted lines represent the percentage of positive cells in unstimulated wells (culture medium only). Data represent mean + SEM of vaccinees (n=10) and control (n=5) volunteers; p-values represent differences between vaccinees and controls by Mann-Whitney.



Supplementary figure 3: Lymphocyte sub-populations.

Representative FACS plots (**a-c**) and relative proportion (**d-f**) of lymphocyte sub-populations in PBMCs isolated from vaccinees (grey bars) or control volunteers (white bars) prior to 1st immunization (day I-1) or prior to challenge (day C-1). **a+d**: Lymphocyte phenotypes: naïve T cells (CD3⁺CD45RO⁻, vertically lined bars), memory T-cells (CD3⁺CD45RO⁺, hashed bars) and non-T-lymphocytes (CD3⁻CD45RO⁻, plain bars); note that all CD45RO⁺ cells are also CD3⁺ (T-cells). **b+e**: T-cell phenotypes: T-helper cells (CD4⁺CD8⁻, vertically lined bars), cytotoxic T-cells (CD4⁺CD8⁺, horizontally lined bars) and other lymphocytes (CD4⁻CD8⁻, plain bars). **c+f**: Memory phenotypes: naïve T-cells (CD62L⁺CD45RO⁻, vertically lined bars), central memory T-cells (CD62L⁺CD45RO⁺, hashed bars), effector memory T-cells (CD62L⁻CD45RO⁺, horizontally lined bars) and other lymphocytes (CD62L⁻CD45RO⁻, plain bars). Data represent mean + SEM of vaccinees (n=10) and control volunteers (n=5).