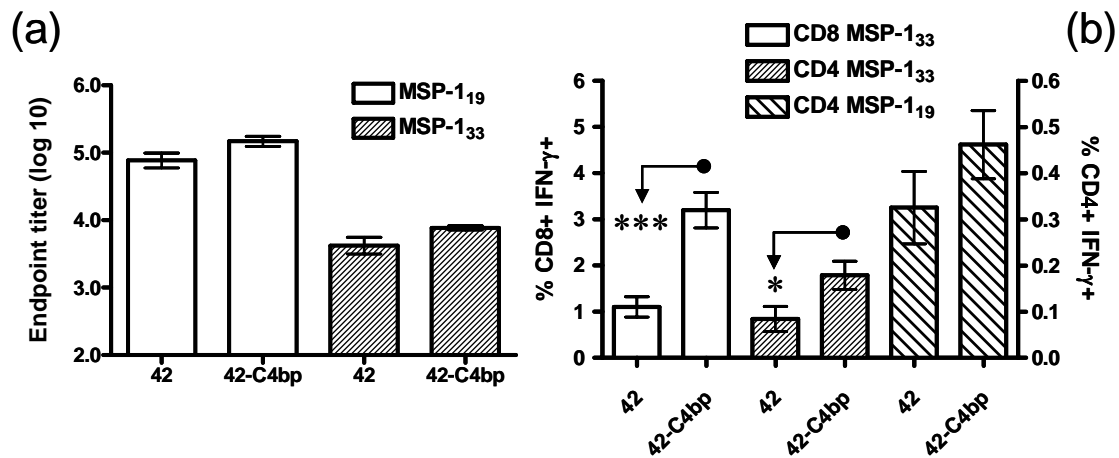# Supplementary Table 1

Immunization Regime (Prime-Boost Interval)	No. Protected / No. Challenged	% Protection	Geometric mean (Range) Peak Parasitemia of Protected Mice
(a) AdHu5-MVA-MSP-1$_{42}$ +/- C4bp in BALB/c mice.			
AdM42 (2wk)	0/6	0%	N/A
AdM42 (8wk)	4/5 + 4/6 + 5/6	76%	0.51% (0.004% - 27.7%)
AdM42-C4bp (8wk)	6/6 + 11/11	100%	0.1% (0% - 9.1%) †
Naïve unimmunized	0/6 + 0/6 + 0/6	0%	N/A
(b) *In vivo* T cell depletion in AdM42-C4bp (8wk) immunized BALB/c mice.			
No depletion	5/5	100%	0.07% (0% - 2.4%)
CD4$^+$ T cell depletion	5/5	100%	0.17% (0% - 0.95%)
CD8$^+$ T cell depletion	5/5	100%	0.26% (0% - 0.26%)
(c) C57BL/6 mice.			
AdM42 (8wk)	6/6	100%	13.9% (3.7% - 56.4%)
AdM42-C4bp (8wk)	6/6	100%	6.7% (2.2% - 13.8%)
Naïve unimmunized	0/6	0%	N/A

Table 1. Protection against *P. yoelii* malaria challenge. Mice were immunized and challenged with 10^4 *P. yoelii* pRBCs. The strain of mouse, immunization regime employed and numbers protected are indicated. In protected groups the geometric mean of peak blood-stage % parasitemias is indicated for those mice which survived the challenge and developed patent parasitemia, and the range of peak blood-stage % parasitemias is indicated for all mice which survived. † 10/17 mice in this group showed sterile immunity (as defined by the absence of patent parasitemia over the 30 day period following challenge).

Supplementary Figure 1. Immunogenicity of AdM42 and AdM42-C4bp immunization in C57BL/6 mice. Mice were immunized with either the AdM42 or AdM42-C4bp regimes as stated in the text. Immune responses were assessed 14 days post-boost. **(a)** Total IgG serum antibody responses against MSP-1$_{19}$ and MSP-1$_{33}$. **(b)** CD8^{+} and CD4^{+} T cell IFN-γ responses following re-stimulation with MSP-1$_{33}$ or MSP-1$_{19}$ peptides were assessed in the spleen by ICS. The mean responses ± SEM are shown ($n \geq 6$ mice per group). $*$ $P \leq 0.05$ and $***$ $P \leq 0.001$ in **b** (independent *t*-test comparing responses between the two groups).

Supplementary Figure 1

SUPPLEMENTARY METHODS

Generation of Recombinant MVA and AdHu5 Vaccines expressing MSP-1$_{42}$

P. yoelii YM MSP-1$_{42}$ (amino acids (aa) 1394-1757) was amplified by Expand PCR (Roche, Lewes, East Sussex, UK) using the 42F forward primer 5'-GTC GAC TCC GAA GAT GCA CCA GAA AAA GAT AT-3' and the 42R reverse primer 5'-GCA TGC GGA TCC TCA GTC TAG ACC TAG CAA AGG GTT AGG AAT TCC CAT AAA GCT GGA AGA ACT ACA GAA TAC-3' from plasmid λPyM4.3[1]. 42R also encodes a C-terminal anti-PK monoclonal antibody recognition sequence IPNPLLGLD. All PCR products were verified by sequence analysis. The human tissue plasminogen activator (tPA) leader sequence was similarly amplified from a plasmid template using the forward primer 5'-GGA TCC GCG CGC CGC CAC C-3' and the reverse primer 5'-CTC GAG TCT TCT GAA TCG GGC ATG G-3'. The forward primer includes the Kozak sequence (underlined). The MSP-1$_{42}$-PK construct was cloned to create an in-frame fusion with the N-terminal tPA leader sequence and cloned into the MVA shuttle vector, pMVA.GFP. This vector, pMVA.GFP.MSP-1$_{42}$, drives antigen expression using the vaccinia P7.5 early/late promoter, and expression of Green Fluorescent Protein (GFP) from the fowlpox late promoter, FP4b. Primary chicken embryo fibroblasts (CEFs, SPF grade) were infected with MVA.Red, which expresses Red Fluorescent Protein (RFP) driven by the vaccinia P7.5 promoter, integrated at the TK locus of MVA. Infected cells were transfected with linearized pMVA.GFP.MSP-1$_{42}$ using Lipofectin (Invitrogen, Paisley, UK). CEFs infected with recombinants expressing GFP (in which MSP-1$_{42}$ and GFP had replaced RFP at the TK locus following homologous recombination) were enriched from those infected with MVA-RFP using a fluorescence-activated cell sorter. Pure

recombinant virus was isolated by repeated plaque picking in CEFs using the GFP marker as visualized by fluorescence microscopy.

Recombinant AdHu5 vaccines were constructed and grown using the ViraPower Adenoviral expression system, as per manufacturer's instructions (Invitrogen). The 1.9 kbp CMV promoter (with regulatory element, enhancer and intron A), polylinker and BGH poly(A) transcription termination sequence from the DNA vaccine vector pSG2[2] was cloned into the pENTR4 vector (Invitrogen), and the MSP-1$_{42}$ construct was then cloned into the polylinker of the CMV promoter, before recombination into the 36 kbp E1- and E3-deleted pAd/PL-DEST AdHu5 genome vector (Invitrogen). Recombinant adenovirus was purified using the Adenopure Kit (PureSyn, Malvern, Pennsylvania, USA). Absorbance at 260nm of virus stock (diluted 1:100 in water) was measured using a UV spectrophotometer, and the viral particle (vp) count was calculated as: Abs_{260} x 10^{12} x dilution factor = vp/mL.

Generation of Recombinant MVA and AdHu5 Vaccines expressing MSP-1$_{42}$-C4bp

The α-chain core domain of mC4bp (GenBank Accession P08607, aa E416 – H469) was cloned into the pMVA.GFP.MSP-1$_{42}$ vector by PCR. The final three amino acids of the original MSP-1$_{42}$ construct (aa 1755-1757) were excluded, as was the C-terminal PK epitope sequence. Recombinant MVA and AdHu5 were generated as described.

Generation of Recombinant MVA and AdHu5 Vaccines expressing *P. falciparum* MSP-1

A composite construct of *P. falciparum* MSP-1, termed PfM128, was designed to include from N- to C- terminus: the conserved blocks[3] 1 (aa 1-55), 3 (aa 162-331), 5 (aa 369-399) and 12 (aa 1079-1170) from strain 3D7 MSP-1 (GenBank Accession NP_704838); followed by blocks 16 and 17 (MSP-1$_{42}$) (aa 1257-1621) from the Wellcome strain (GenBank Accession X02919)[4]; followed by a glycine-proline linker (GGGPGGG); followed by blocks 16 and 17 (MSP-1$_{42}$) (aa 1320-1709) from strain 3D7 MSP-1. Three amino acid substitutions were included in both alleles of Block 17/MSP-1$_{19}$ in order to abolish blocking antibody epitopes whilst maintaining inhibitory antibody epitopes[5], aid antigen processing[6], and remove an N-glycosylation site: S1609 to A, C1618 to I, and C1634 to W for 3D7; and S1528 to A, C1537 to I, and C1553 to W for Wellcome. The final construct is codon optimized for human expression and was synthesized by GeneArt GmbH (Regensburg, Germany). To minimize any chance of homologous recombination between the highly similar coding sequences for the two alleles of MSP-1$_{19}$, codon optimization included minimum homology at the nucleotide sequence level for these two sequences (final base pair sequences are 74.5% similar). The PfM128 construct was cloned into the pMVA.GFP and pENTR4 vectors as described above to generate recombinant viruses.

Animals and Immunizations

Female BALB/c (H-2^d) and C57BL/6 (H-2^b) mice (BMSU, John Radcliffe Hospital, Oxford, UK), 6-8 weeks old, were used in all experiments. All procedures were carried

out under the terms of the UK Animals (Scientific Procedures) Act Home Office Project

Licence. Mice were immunized intradermally (i.d.) with 5 x 10^7 pfu MVA vaccines,

unless otherwise stated in the text, or 5 x 10^{10} vp AdHu5 vaccines, diluted in endotoxin-

free phosphate-buffered saline (PBS) (Sigma, Poole, Dorset, UK).

Generation of Recombinant GST-Fusion Proteins

P. yoelii YM MSP-1$_{19}$ (aa 1649-1757) was amplified by PCR as above using the 19F

forward primer 5'-GGA TCC GTC GAC ATG GAT GGT ATG GAT TTA TTA GGT G-

3' and the 42R reverse primer and cloned into the glutathione *S*-transferase (GST)-fusion

protein expression vector pGEX-2T (Amersham Biosciences, Bucks., UK), before

transformation into Rosetta *Escherichia coli* cells (Novagen, Nottingham, UK). The

proteins were produced as described previously[7] with some modifications. Cells were

harvested and lysed using BugBuster and benzonase endonuclease (Novagen), and then

ultracentrifuged. Recombinant protein was purified by affinity chromatography using the

GST·Bind Purification Kit (Novagen) as per manufacturer's instructions. Recombinant

GST-MSP-1$_{33}$ and GST control[7] were a kind gift from Miss I. T. Ling (NIMR, London,

UK).

P. yoelii **MSP-1 ELISA**

Serum from mice was analyzed for antibodies by indirect ELISA. Recombinant GST-

MSP-1 fusion protein or GST control were adsorbed overnight at room temperature (RT)

to 96 well Nunc-Immuno Maxisorp plates (Fisher Scientific, Wohlen, Germany) at

2µg/mL in PBS. Sera were typically diluted to 1:100, added in duplicate wells and

serially diluted. Bound antibodies were detected using alkaline phosphatase-conjugated goat anti-mouse total IgG (Sigma) or biotin-conjugated rat anti-mouse IgG1, IgG2a, IgG2b or IgG3 (BD Biosciences, Oxford, UK) followed by incubation with ExtrAvidin alkaline phosphatase conjugate (Sigma). Plates were developed by adding *p*-nitrophenylphosphate substrate (Sigma). Optical density was read at 405nm (OD_{405}). Endpoint titers were taken as the x-axis intercept of the dilution curve at an absorbance value 3x standard deviations greater than the OD_{405} for naïve mouse serum (typical cut-off OD_{405} for positive sera = 0.15). A standard serum sample was included in all assays as a reference control. All GST control ELISAs were negative (data not shown).

Intracellular Cytokine Staining (ICS)

Specific IFN-γ secretion by mouse splenocytes was assayed by ICS. Splenocytes were re-stimulated in the presence of GolgiPlug (BD Biosciences) for 5h at 37ºC with pools of 15-mer peptides overlapping by 10 aa (final concentration 5μg/mL each peptide). Overlapping peptide pools corresponded to MSP-1$_{33}$ (aa 1394-1663) containing 52 peptides, and MSP-1$_{19}$ (aa 1654-1757) containing 19 peptides. Staining antibodies were purchased from eBiosciences. After blocking Fc receptors with anti-CD16/CD32, cells were surface stained for 30min at 4ºC with Pacific Blue-labelled anti-CD8α (clone 53-6.7) and APC-Alexa Fluor 750-labelled anti-CD4 (clone RM4-5). Cells were permeabilized in Cytofix/Cytoperm solution as per manufacturer's instructions (BD Biosciences). Intracellular IFN-γ was stained with PE-labelled anti-mouse IFN-γ (clone XMG1.2). Samples were analyzed using a CyAn ADP Flow Cytometer (Dako, Ely, UK) and FlowJo software. Data are shown as the % of total $CD4^{+}$ or $CD8^{+}$ cells that stain

positive for IFN-γ. Background responses in unstimulated control cells were all ≤ 0.05%, and these values were subtracted from the stimulated response.

In vivo Depletions

In vivo depleting monoclonal antibodies (mAbs), were purified by protein G affinity chromatography from hybridoma culture supernatants. Anti-CD4 GK1.5 (rat IgG2a) and anti-CD8 2.43 (rat IgG2a) were diluted in sterile PBS. Normal rat IgG (nRatIgG) was purchased from Sigma and purified by the same method. For depletion of CD4$^+$ or CD8$^+$ T cells, mice were injected intraperitoneally (i.p.) with 200μg of the relevant mAb on days -2 and -1 before, and on the day of challenge. Mice were further administered the same dose of mAbs on days +7, +14 and +21 post-challenge. The degree of *in vivo* T cell depletion was assessed by flow cytometry of surface-stained splenocytes from depleted and control mice. Cells were surface-stained for CD4 and CD8 as above, and also with FITC-labelled anti-mouse CD3ε (clone 145-2C11). Flow cytometric analysis indicated *in vivo* depletion to be >95% effective at the time of challenge (data not shown).

P. yoelii pRBC Challenge

P. yoelii parasites (strain YM) were kindly provided by Dr G. A. Butcher (Imperial College, London, UK). For blood-stage challenge, mice were infected with 10^4 parasitized red blood cells (pRBCs) by the intravenous (i.v.) route. Blood-stage parasitemia was monitored from day two post-challenge by microscopic examination of Giemsa-stained blood smears. Mice were deemed uninfected in the absence of patent

parasitemia in 50 fields of view. Infection was considered lethal when parasitemia exceeded 80%, at which point animals were euthanized.

Standardized ELISA against *P. falciparum* MSP-1$_{42}$.

Measurement of anti-MSP-1$_{42}$ (3D7 or FVO) antibodies was performed by the GIA Reference Center (LMVR, NIH) according to a standardized ELISA protocol[8]. ELISA plates were coated with 100ng per well of MSP1$_{42}$ (3D7 or FVO) protein[9]. A mouse anti-MSP-1$_{42}$ standard serum was made using a pool of sera from immunized mice and this standard pool was assigned a value approximately equivalent to the reciprocal of the dilution giving an optical density (OD) of 1.0 at a wavelength of 405nm. Duplicates of the serially-diluted standard serum pool were included on the test plates in order to generate a standard curve. The standard curve was fitted to a four parameter hyperbolic function, which was used to convert the absorbance of individual test sera into antibody units (SOFTmax PRO version 4, Molecular Devices Co.).

In vitro* Growth Inhibition Assay of *P. falciparum

A standardized *in vitro* growth inhibition assay against the 3D7 and FVO strains of *P. falciparum* was carried out by the GIA Reference Center (LMVR, NIH) as previously described[10]. Briefly, serum was pooled from unimmunized control and immunized mice and total IgG was purified using protein G columns (PIERCE, Rockford, IL) according to the manufacturer's instructions. The eluted IgGs were dialyzed against RPMI 1640 and concentrated to 10mg/mL and subsequently sterilized with a 0.22μm filter (Millipore). The purified IgGs were pre-adsorbed with uninfected human O$^+$ RBCs (25mL of RBCs

per 1mL of sample) for 1h to remove anti-human RBC immunoglobulins. For the assay itself, 50μL test samples or controls, synchronized *P. falciparum* parasites (late trophozoites and schizonts) and culture medium were applied as a total volume of 100μL per well into 96-well tissue culture plates and tested in triplicate. The final concentration of the culture was 0.3 ± 0.1% parasitemia, 1% hematocrit in growth medium (RPMI 1640 containing 10% human O^+ serum, 25mM HEPES, 0.4mM hypoxanthine, 30mM sodium bicarbonate and 25mg/L of gentamicin). The cultures were maintained for 40 to 42h and relative parasitemia levels were quantified by biochemical determination of parasite lactate dehydrogenase (LDH). Percent inhibition of the immune IgG was calculated as 100 - [(A_{650} of test IgG - A_{650} of normal RBCs)/(A_{650} of infected RBCs without any IgG - A_{650} of normal RBCs) x 100].

Statistical Analysis

Data were analyzed using GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, California, USA). Data set normality was assessed by Kolmogorov-Smirnov test. ELISA endpoint titers were normalized by log_{10} transformation. Independent-samples or paired two-tailed Student's *t*-test was performed, as appropriate, to compare mean immune responses between two groups. Survival rates were compared using chi-squared tests. A value of $P \leq 0.05$ was considered significant in all cases.

SUPPLEMENTARY REFERENCES

1.   Lewis, A.P. *Mol Biochem Parasitol* **36**, 271-82 (1989).

2.   McShane, H. et al. *Infect Immun* **69**, 681-6 (2001).

3.   Miller, L.H. et al. *Mol Biochem Parasitol* **59**, 1-14 (1993).

4.   Holder, A.A. et al. *Nature* **317**, 270-3 (1985).

5.   Uthaipibull, C. et al. *J Mol Biol* **307**, 1381-94 (2001).

6.   Hensmann, M. et al. *Eur J Immunol* **34**, 639-48 (2004).

7.   Ahlborg, N. et al. *Infect Immun* **70**, 820-5 (2002).

8.   Miura, K. et al. *Vaccine* **26**, 193-200 (2008).

9.   Malkin, E. et al. *PLoS Clin Trials* **2**, e12 (2007).

10.   Mullen, G.E. et al. *Vaccine* **24**, 2497-505 (2006).