## 3. SUPPLEMENTAL MATERIALS & METHODS

### Study Design, Participants, Vaccination and Follow-up

The study was conducted at the Oxford Vaccine Centre, part of the Centre for Clinical Vaccinology and Tropical Medicine, University of Oxford. All volunteers gave written informed consent prior to participation. The study was conducted according to the principles of the Declaration of Helsinki and in accordance with Good Clinical Practice (GCP). Approvals were granted by the UK Gene Therapy Advisory Committee (GTAC 166) and the UK Medicines and Healthcare products Regulatory Agency (Ref: 21584/0253/001-0001). Vaccine use was authorized by the Genetically Modified Organisms Safety Committee (GMSC) of the Oxford Radcliffe Hospitals NHS Trust (Reference number GM 462.09.43). The trial was registered with ClinicalTrials.gov (Ref: NCT01003314). A Local Safety Monitor provided safety oversight, whilst GCP compliance was externally monitored.

Healthy malaria-naïve adults aged 18 - 50 years old were recruited from the Oxford area from November 2009 to January 2010. Volunteers were not screened for pre-existing neutralizing antibodies (NAb) to the ChAd63 vector [1], and there was no selection of volunteers on the basis of low NAb titers. Five volunteers were excluded following screening for the following reasons; β-thalassemia trait, unexplained eosinophilia, anaemia, abnormal abdominal examination and travel to a malaria endemic region in the six months preceding study enrolment. Six volunteers (Group 1) were vaccinated with $5 \times 10^9$ viral particles (vp) ChAd63 MSP1 intramuscularly (i.m.) and four of these were subsequently vaccinated with $5 \times 10^8$ plaque forming units (pfu) MVA MSP1 i.m. 56 days later. Another ten volunteers (Group 2) were vaccinated with $5 \times 10^{10}$ vp ChAd63 MSP1 i.m. and eight of these were subsequently vaccinated with $5 \times 10^8$ pfu MVA MSP1 i.m. 56 days later (**Fig. 1**). The three

volunteers in Group 2C were subsequently recruited into a Phase IIa challenge safety study (Sheehy SH et al., manuscript in preparation) at the day 84 time-point. Safety and immunogenicity data are included for these three volunteers up until that time-point.

**Inclusion Criteria**

- Healthy adult aged 18 to 50 years.
- Able and willing (in the Investigator's opinion) to comply with all study requirements.
- Willing to allow the investigators to discuss the volunteer's medical history with their General Practitioner.
- For females only, willingness to practice continuous effective contraception during the study.
- For males only, willingness to use barrier contraception until 3 months after last vaccination.
- Agreement to refrain from blood donation during the course of the study.
- Written informed consent.

**Exclusion Criteria**

Volunteers were excluded from the study if any of the following criteria were met:

- Any history of malaria.
- Travel to a malaria endemic region during the study period or within the six months preceding enrolment.
- Significant concern raised by General Practitioner in relation to participation.
- Participation in another research study involving an investigational product in the 30 days preceding enrolment, or planned use during the study period.
- Prior receipt of a recombinant adenoviral and/or MVA-vectored vaccine.
- Administration of immunoglobulins and/or any blood products within the three months preceding the planned administration of the vaccine candidate.
- Confirmed or suspected immunosuppressive or immunodeficient state, including HIV infection; asplenia; recurrent, severe infections and chronic (more than 14 days)

immunosuppressant medication use within the 6 months preceding enrolment (excluding inhaled and topical steroids).

- History of allergic disease or reactions likely to be exacerbated by any component of the vaccine, e.g. egg products, Kathon.
- History of clinically significant contact dermatitis.
- A predicted ten year risk of fatal cardiovascular disease of ≥5%, as estimated by the Systematic Coronary Risk Evaluation (SCORE) system.
- History of arrhythmia or congenital QT interval prolongation.
- Family history of sudden cardiac death.
- Contraindication to both Riamet and chloroquine.
- Concomitant use of other drugs known to cause QT-interval prolongation, (e.g. macrolides, quinolones, amiodarone).
- History of anaphylaxis in reaction to vaccination.
- Pregnancy, lactation or willingness/intention to become pregnant during the study.
- History of cancer (except basal cell carcinoma of the skin and cervical carcinoma in situ).
- History of serious psychiatric illness.
- Any other serious chronic illness requiring hospital specialist supervision.
- Suspected or known current alcohol abuse as defined by an alcohol intake of greater than 42 units every week.
- Suspected or known injecting drug abuse.
- Seropositive for hepatitis B surface antigen (HBsAg).
- Seropositive for hepatitis C virus (antibodies to HCV).
- Any clinically significant abnormal finding on screening biochemistry or haematology blood tests or urinalysis.
- Any other significant disease, disorder or finding, which, in the opinion of the Investigator may either put the volunteer at risk because of participation in the study, or may influence the result of the study, or the volunteer's ability to participate in the study.

**Re-vaccination exclusion criteria**

Anaphylactic reaction following administration of vaccine and pregnancy were absolute contraindications to further administration of vaccine.

**Safety Assessment**

The primary objective of this trial was to assess the safety of the ChAd63 and MVA vaccines expressing MSP1. Volunteers were observed for 2 h post 5 x $10^9$ vp ChAd63 MSP1 immunization, 1 h post 5 x $10^{10}$ vp ChAd63 MSP1 and 30 min post 5 x $10^8$ pfu MVA MSP1. Volunteers were given a digital thermometer, injection site reaction measurement tool and symptom diary card to record their daily temperature, injection site reactions and solicited adverse events for 14 days following vaccination with ChAd63 MSP1 and 7 days following vaccination with MVA MSP1. Local and systemic reactogenicity was evaluated at subsequent clinic visits and graded for severity, outcome and association to vaccination as per the criteria outlined in Tables S1-4. Blood was sampled at all visits post vaccination except days 2 and 58, and the full blood count with differential, platelet count and serum biochemistry (including electrolytes, urea, creatinine, bilirubin, alanine aminotransferase, alkaline phosphatase and albumin) measured.

**Vaccines**

The ChAd63 and MVA viral vaccines encode an insert (previously termed PfM128) that is composed of the conserved blocks of sequence (1, 3, 5, and 12) from *P. falciparum* MSP1 followed by both of the known allelic sequences encoding the 42kDa C-terminus fused in tandem [2] (**Fig. S1**). These were manufactured under Good Manufacturing Practice conditions by the Clinical Biomanufacturing Facility, University of Oxford (ChAd63 MSP1), and IDT Biologika, Rosslau, Germany (MVA MSP1). Briefly, ChAd63 MSP1 was generated in suspension HEK293 cells and purified by caesium chloride density-gradient centrifugation. MVA MSP1 was generated in chicken embryo fibroblasts (CEFs) and purified by sucrose density-gradient centrifugation. Each vaccine lot underwent comprehensive quality control analysis to ensure that the purity, identity, and integrity of the virus met pre-defined

specifications. Vaccine lots were stored at the clinical site at -80°C and the temperature was monitored. ChAd63 MSP1 vaccine stability was monitored by using an infectivity assay in HEK 293 cells. The immuno-potency of the MVA MSP1 vaccine was confirmed by regular immunogenicity evaluation in mice.

**PBMC and Serum Preparation**

Blood samples were collected into lithium heparin-treated vacutainer blood collection systems (Becton Dickinson, UK). PBMC were isolated and used within 6 h as previously described [3]. Excess cells were frozen in FCS containing 10% DMSO and stored in liquid nitrogen. If required, frozen cells were thawed in a water bath at 37°C and immediately transferred into warm R10 medium. Cells were washed twice in warm R10, resuspended in R10 containing 25U/mL benzonase (Sigma) and incubated for 4 h at 37°C, 5% $CO_2$ in a humidified incubator. Live cells were counted by trypan blue staining, washed and resuspended in fresh R10 and used in the assay. For serum preparation, untreated blood samples were stored at 4°C and then the clotted blood was centrifuged for 5 min (1000 *xg*). Serum was stored at -80°C.

**Peptides for T cell Assays**

Peptides were purchased from NEO Peptide, USA. The peptides, 20 amino acids (aa) in length and overlapping by 10 aa covered the entire MSP1 insert present in the viral vectored vaccines. Peptides were reconstituted in 100% DMSO at 50-200 mg/mL and combined into various pools for ELISPOT and ICS assays as described in Table S5.

**Ex-vivo IFN-γ ELISPOT**

Fresh PBMC were used in all ELISPOT assays using a previously described protocol [4], except that 50µL/well MSP1 peptide pools (Table S5) (final concentration each peptide 5µg/mL) were added to test wells, 50µL/well R10 and DMSO control were added to negative unstimulated wells, and 50µL/well Staphylococcal enterotoxin B (SEB) (final concentration 0.02µg/mL) plus phytohemagglutinin (PHA) (final concentration 10µg/mL) was added to positive control wells. Spots were counted using an ELISPOT counter (Autoimmun Diagnostika (AID), Germany). Results are expressed as IFN-γ spot-forming units (SFU) per million PBMC. Background responses in unstimulated control wells were almost always less than 20 spots, and were subtracted from those measured in peptide-stimulated wells.

**Multiparameter Flow Cytometry**

Cytokine secretion by PBMC was assayed by intracellular cytokine staining (ICS) followed by flow cytometry. Frozen PBMC were re-stimulated in 200µL aliquots of 1 x $10^6$ cells in a 96-well U-bottom plate. Re-stimulation was carried out for 18 h at 37ºC in R10 medium in the presence of 1µg/mL each of mouse anti-human CD49d and CD28 (BD Biosciences) and 10µL CD107a-PE-Cy5 (clone H4A3). Re-stimulation for the final 16 h was carried out in the presence of 10µg/mL Brefeldin A (Sigma) and 0.7µg/mL Monensin (GolgiStop, BD Biosciences). Each sample was re-stimulated with either: 2µg/mL SEB (positive control samples); a pool of all 109 peptides spanning the MSP1 vaccine antigen (see Table S5) at final concentration 2µg/mL each peptide and 0.21% total DMSO concentration; 0.21% DMSO final concentration (unstimulated peptide control sample); cryopreserved red blood cells infected with schizont / late trophozoite stage 3D7 strain *P. falciparum* parasites (iRBC) at 5 x $10^6$/mL; or uninfected red blood cell controls (uRBC) at 5 x $10^6$/mL prepared in the same manner. Cells were stained the next day in three steps (30min at 4ºC for each step).

Staining antibodies were rat or mouse anti human IgG mAb purchased from eBiosciences or BD Biosciences. Cells were surface stained in FACS buffer (PBS with 0.01% sodium azide and 0.1% BSA) and washed in between each step: step i) LIVE/DEAD Fixable Aqua Dead Cell Stain Kit for 405nm excitation (Invitrogen); step ii) CD4 eFluor605 (clone OKT4), CD14-eFluor450 (clone 61D3), CD20-Pacific Blue (clone 2H7). Cells were then fixed and permeabilized in Cytofix/Cytoperm (BD Biosciences) for 10 min at 4°C, before intracellular staining in Permwash with CD8α APC-eFluor780 (clone RPA-T8), CD3-Alexa Fluor 700 (clone SP34-2), IFN-γ-FITC (clone 4S.B3), TNFα-PE-Cy7 (clone MAb11), and IL-2-PE (clone MQ1-17H12). Samples were washed, resuspended in PBS and analyzed using a LSRII Flow Cytometer (BD Biosciences) and FlowJo v8.8 (Tree Star Inc, USA). Single stained compensation beads (BD Biosciences) were used as controls to automatically calculate compensation. Dead cells, monocytes ($CD14^+$), and B cells ($CD20^+$) were excluded from the analysis. The boolean gate platform was used with individual gates to create response combinations (**Fig. S9**). Analysis and presentation of distributions was performed using SPICE v5.1, downloaded from http://exon.niaid.nih.gov/spice [5]. Background responses in unstimulated peptide and uRBC control cells were subtracted from the MSP1 peptide and iRBC stimulated responses respectively.

**Total IgG ELISA**

The production in *E. coli* and purification of recombinant GST-PfMSP1$_{19}$ (either ETSR 3D7/Mad20 allele or the QKNG Wellcome/K1 allele) or GST control protein has been described elsewhere [2]. Protein was coated onto 96 well Nunc-Immuno Maxisorp plates at a concentration of 2μg/mL in PBS and left over-night. The next day plates were washed 6x in PBS containing 0.05% Tween 20 (PBS/T) and blocked for 1h with Casein block solution (Pierce, UK). Plates were washed again, and then a standard, test sera, internal control and

blank samples all diluted in Casein block solution were added to each plate for 2h according to published methodology [6]. The standard was prepared from adult Kenyan immune serum (a kind gift from Dr Faith H. Osier, KEMRI-Wellcome, Kilifi, Kenya) and was serially diluted on every plate to make a standard curve. Plates were washed again, followed by addition for 1h of alkaline phosphatase-conjugated goat anti-human IgG (γ-chain) (Sigma) diluted 1:1000 in Casein block solution. Plates were washed again and bound antibodies were detected by adding *p*-nitrophenylphosphate substrate (Sigma) diluted in diethanolamine buffer (Fisher Scientific, UK). Optical density was read at 405nm ($OD_{405}$) using an ELx800 microplate reader (BioTek, UK). The ELISA unit value of the standard was assigned as the reciprocal of the dilution giving an $OD_{405}$ of 1.0 in the standardized assay. The $OD_{405}$ of individual test samples was converted into ELISA units by using the standard curve and Gen5 ELISA software v1.10 (BioTek, UK). All sera tested against the GST control protein were less than the minimal detection level of the assay (data not shown). Antibodies against $PfMSP1_{42}$ (3D7 and FVO alleles) were assayed by the GIA Reference Center, NIH, USA as previously described [6], and these OD-based ELISA units were converted to μg/mL also as described previously [7]. Antibodies against the $PfMSP1_{83}$, $PfMSP1_{30}$, $PfMSP1_{38}$ and $PfMSP1_{42}$ regions of MSP1 were assayed as previously described [8].

**Immunofluorescence Assay (IFA)**

*P. falciparum* (3D7 strain) parasites were cultured on intact $O^+$ erythrocytes. Cultures of high parasitemia (>10%) and a high synchrony of late stages (schzionts and late trophozoites) were smeared onto glass microscope slides and stored at -20°C. For detection of immunofluorescence, slides were fixed for 15 min in 4% paraformaldehyde, permeabilized for 10 min in 0.1% Triton X100 and then quenched for 5 min in 0.01% sodium borohydride. Slides were then blocked in Casein block solution for 1 h. Volunteer sera were diluted 1:100

in Casein block solution and pre-adsorbed with uninfected $O^+$ human erythrocytes for 45 minutes (approx. 5 x $10^4$ erythrocytes per μl of diluted serum). A negative control was included, consisting of a pool of pre-immunization sera. A positive control was included consisting of a human $PfMSP1_{19}$-specific monoclonal antibody (mAb) (a human IgG3 recognizing epitope C1 [9]) tested at 2μg/mL (a kind gift from Prof Richard Pleass, Liverpool School of Tropical Medicine, Liverpool, UK). Samples were allowed to bind for 30 min, before the addition of goat anti-human IgG-Alexa 488 conjugate (Invitrogen, UK). DNA was counterstained with 4',6-diamidino-2-phenylindole (DAPI), and the slides were mounted and viewed under a Leica DMI3000 microscope. Day 84 sera from all 12 volunteers in Groups 1B and 2B+C tested positive, but representative results are shown (**Fig. 5d**).

***In vitro* Growth Inhibitory Activity (GIA) Assay**

The ability of induced anti-MSP1 antibodies to inhibit growth of *P. falciparum* 3D7 parasites was assessed by a standardized GIA assay using purified IgG as previously described [7, 10]. Briefly, each test IgG (10mg/mL in a final test well) was incubated with synchronized *P. falciparum* parasites for ~48h and relative parasitemia levels were quantified by biochemical determination of parasite lactate dehydrogenase.

**Supplementary Safety & Reactogenicity Data**

1. **ChAd63 MSP1:**

69% of volunteers experienced one or more local injection-site reaction(s) which were mild in intensity, with the exception of one case of moderate arm pain post ChAd63 MSP1 5 x $10^{10}$ vp. 94% of volunteers experienced one or more systemic AEs. These were mild in intensity with the exception of a headache of moderate severity starting on day of vaccination and lasting 2 days post ChAd63 MSP1 5 x $10^{10}$ vp.

**Figure 2a,b:** 'Other' AEs post 5 x $10^9$ vp ChAd63 MSP1 included mild thrombocytopenia, coryzal symptoms, diarrhoea and hordeolum. 'Other' AEs post 5 x $10^{10}$ vp ChAd63 MSP1 included sore throat, two cases of coryzal symptoms, mild thrombocytopenia & elevated ALT (69 IU/L).

2. **MVA MSP1:**

Two volunteers developed moderate or severe erythema post vaccination (**Fig. 2c**). Volunteer (i): Day 3 post vaccination with MVA MSP1 5 x $10^8$ pfu, a 6 x 4 cm raised, circular, non-tender erythematous rash developed 8 cm distal to the vaccination site. There was no history of trauma and the volunteer's mild systemic AEs post vaccination had fully resolved by the time the rash developed. The volunteer described a past history of generalized urticarial 'hives' at times of stress, however had never experienced an isolated 'hive' or a rash similar to that seen post MVA MSP1. The rash resolved with no sequelae 8 days later. Volunteer (ii): Day 5 post vaccination with MVA MSP1 5 x $10^8$ pfu, a 6 x 12 cm irregular non-erythematous, non-raised rash developed 3 cm distal to the vaccine site. The volunteer's mild local and systemic AEs post vaccination had fully resolved by the time the rash developed. The rash fully resolved within 2 days. To our knowledge, this reaction has not previously been described post MVA vectored vaccines. **Figure 2c:** Local and systemic AEs post MVA MSP1: mild 'other' AEs post MVA MSP1 included coryzal symptoms, vasovagal episode, "prickly skin", oesophagitis, tender cervical lymphadenopathy and elevated ALT (61 IU/L). Moderate 'other' AEs post MVA MSP1 included bruising at vaccination site. Severe 'other' AEs post MVA MSP1 included two cases of rigors.

## Supplemental References


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