# Translating the Immunogenicity of Prime-boost Immunization With ChAd63 and MVA ME-TRAP From Malaria Naive to Malaria-endemic Populations

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To induce a deployable level of efficacy, a successful malaria vaccine would likely benefit from both potent cellular and humoral immunity. These requirements are met by a heterologous prime-boost immunization strategy employing a chimpanzee adenovirus vector followed by modified vaccinia Ankara (MVA), both encoding the pre-erythrocytic malaria antigen ME-thrombospondin-related adhesive protein (TRAP), with high immunogenicity and significant efficacy in UK adults. We undertook two phase 1b open-label studies in adults in Kenya and The Gambia in areas of similar seasonal malaria transmission dynamics and have previously reported safety and basic immunogenicity data. We now report flow cytometry and additional interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) data characterizing pre-existing and induced cellular immunity as well as anti-TRAP IgG responses. T-cell responses induced by vaccination averaged 1,254 spot-forming cells (SFC) per million peripheral blood mononuclear cells (PBMC) across both trials and flow cytometry revealed cytokine production from both $CD4^+$ and $CD8^+$ T cells with the frequency of $CD8^+$ IFN-γ-secreting monofunctional T cells (previously shown to associate with vaccine efficacy) particularly high in Kenyan adults. Immunization with ChAd63 and MVA ME-TRAP induced strong cellular and humoral immune responses in adults living in two malaria-endemic regions of Africa. This prime-boost approach targeting the pre-erythrocytic stage of the malaria life-cycle is now being assessed for efficacy in a target population.

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## INTRODUCTION

Approximately 1.2 billion people worldwide are at high risk of malaria and there were an estimated 207 million cases of malaria in 2012, with 90% occurring in sub-Saharan Africa and 57% of the African population living in areas of moderate to high transmission intensity.[1,2] Malaria caused by *Plasmodium falciparum* remains one of the leading causes of death in children under 5 years of age in sub-Saharan Africa, despite the deployment of multiple control strategies such as the distribution of insecticide-treated nets, indoor residual spraying, and intermittent preventive treatment.[3] An effective infant vaccine for malaria could substantially reduce the burden of disease and levels of malaria mortality and morbidity. The 2013 Malaria Vaccine Funders Group malaria vaccine roadmap has a landmark goal of producing a first-generation malaria vaccine by 2015 with greater than 50% efficacy against severe disease with greater than 1 year's duration.[4] The current leading malaria vaccine candidate, RTS,S, is undergoing phase 3 trials across Africa in some 16,000 infants, however despite inducing sterile efficacy in a high proportion of malaria-naive adult volunteers,[5] observed efficacy of vaccination with RTS,S/AS02 was only 30% in the target age group of 6–12-week-old infants and 50% among 5–17 month olds in malaria-endemic regions.[6] Immune responses induced by vaccination with RTS,S include both humoral- and T-cell responses, however the cellular immune response is exclusively $CD4^+$ T-cell-mediated and relatively modest.[7,8] Studies of protective efficacy in rodent models

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repeatedly show that high frequencies of $CD8^+$ T cells are essential for sterile protection[9]; this and evidence from animal models[10–12] and studies in humans of vaccination with attenuated sporozoites,[13] suggest that a successful malaria vaccine will benefit from both a humoral and cellular component. Therefore, it is highly likely that an additional component capable of generating high frequencies of $CD8^+$ T cells will be beneficial to complement the protection afforded by RTS,S or any other antibody-inducing malaria vaccine.[14]

The Jenner Institute at Oxford University has been developing a program of viral vectored vaccines against infectious diseases including malaria for the past 14 years, the most promising of which utilizes the full length pre-erythrocytic antigen thrombospondin-related adhesive protein (TRAP) fused to a multi-epitope (ME) string of known T-cell epitopes.[15] In previous studies with the ME-TRAP antigen, initial results in malaria naive volunteers in the United Kingdom were promising with strategies such as priming with a fowlpox vector (FP9) and boosting with modified vaccinia Ankara (MVA),[16] however results of subsequent immunogenicity studies in target populations in Africa were disappointing with immune responses detected at levels only a small fraction of those in naive adult subjects.[17,18] In consecutive studies, responses in adult UK volunteers averaged 475 spot-forming cells (SFC) per million peripheral blood mononuclear cells (PBMC) compared to 389 SFC/$10^6$ PBMC in coastal Kenya, which fell to 105 SFC/$10^6$ PBMC in Kenyan children aged 1–6 years demonstrating the difficulty in translating immunogenicity outcomes between populations.[16,19,20]

More recently, very potent cellular immune responses to ME-TRAP have been elicited by replacing the poxvirus priming vector with a chimpanzee adenovirus (ChAd63).[21–23] The advantages of using chimpanzee adenoviruses rather than human serotypes include an excellent safety profile and enhanced immunogenicity as a consequence of lower levels of pre-existing neutralizing antibodies against the vector in the target populations for vaccination. A phase 1 safety and dose-escalation study

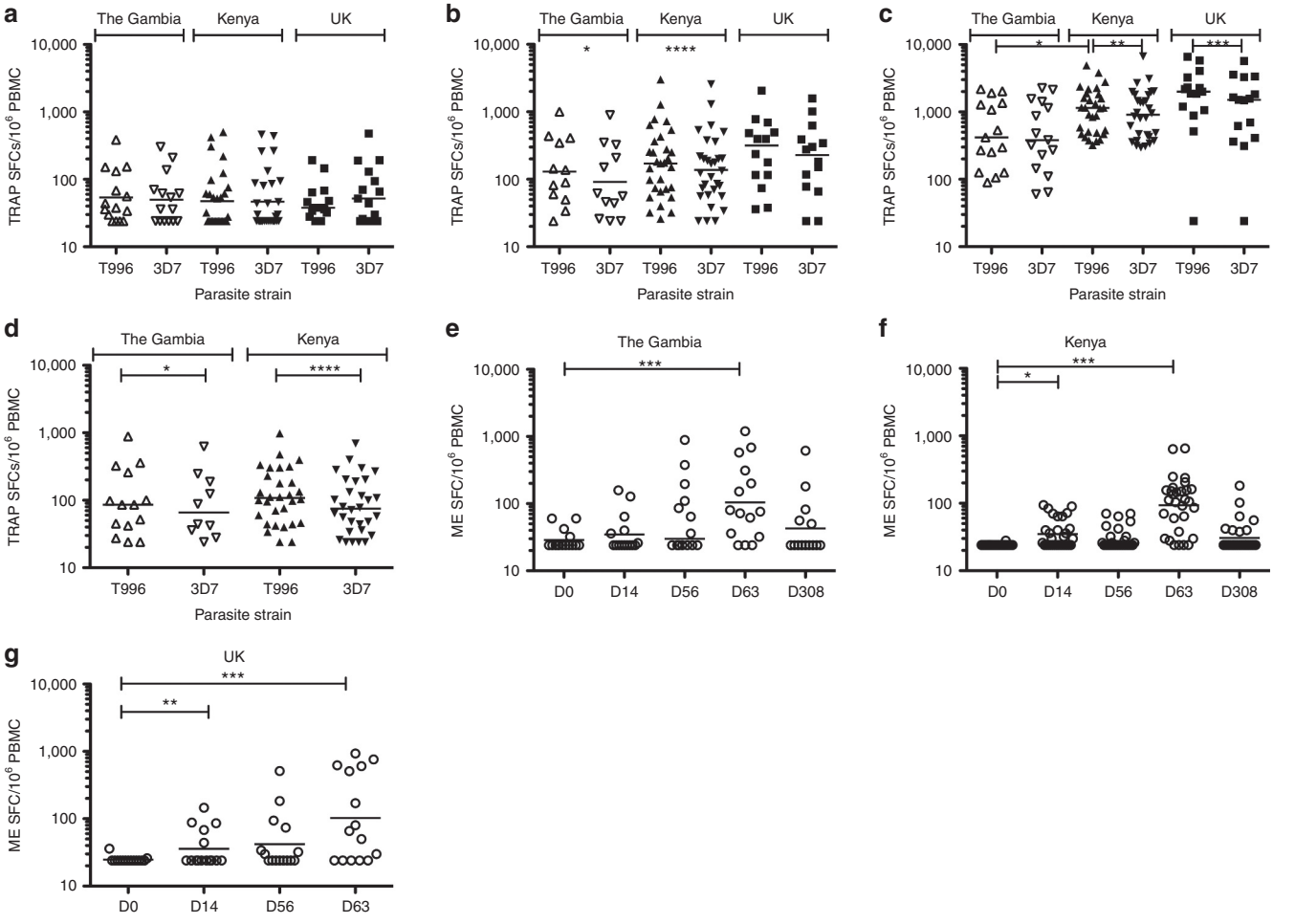


**Figure 1** ***Ex vivo*** **IFN-γ ELISPOT responses pre- and postvaccination to ChAd63 and modified vaccinia Ankara (MVA) ME-TRAP-vectored vaccines in Gambian, Kenyan, and UK volunteers from a controlled human malaria infection trial vaccinated with the same construct.** All volunteers received one dose of ChAd63 ME-TRAP followed by one dose of MVA ME-TRAP 8 weeks later. (**a**) Prevaccination responses to TRAP. (**b**) Responses to TRAP at 14 days postprime with ChAd63, ****$P \leq 0.0001$, $P = 0.03$. (**c**) Responses to TRAP at day 63 (7 days postboost with MVA), ***$P = 0.0002$, **$P = 0.003$, *$P = 0.04$. (**d**) Responses to TRAP at day 300 postprime with ChAd63, ****$P \leq 0.0001$, *$P = 0.03$. (**e–g**) Responses to the ME string in Gambian, Kenyan, and UK volunteers, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. ELISPOT responses to peptides from different strains were compared a using two-tailed Wilcoxon-matched pairs analysis. Increases in ELISPOT responses to ME were compared to responses at day 0 using Kruskall-Wallis with Dunn's multiple comparison posttest. Lines represent medians. ELISPOT, enzyme-linked immunospot; TRAP, thrombospondin-related adhesive protein.

demonstrated a good safety profile for ChAd63 ME-TRAP, and when combined with MVA ME-TRAP in a heterologous prime-boost approach induced T-cell frequencies of more than 2,000 SFC per million PBMC in adult UK volunteers.[21]

Between May 2010 and May 2011, we undertook two phase 1b trials of heterologous prime-boost with ChAd63-MVA ME-TRAP to study the effect of vaccine dose and route of administration on safety and immunogenicity profiles in Kenyan and Gambian adults. The trials were carried out in areas of similar malaria transmission characteristics. Volunteers received a priming vaccination with the simian adenovirus ChAd63, followed by a boosting dose of MVA 8 weeks later, with both vectors encoding ME-TRAP antigen. Previous immunogenicity and efficacy studies with this regimen in UK volunteers showed a good safety profile, very potent T-cell induction, and protection against experimental malaria in a controlled human malaria infection (CHMI) model.[21] Efficacy was observed both through sterilizing immunity in 21% of recipients and a significant delay to patent parasitemia as determined by blood film microscopy in a further 34% of vaccinees, thus giving some degree of efficacy in more than half of vaccinees.[24]

Primary outcomes for these two phase 1b studies in malaria-endemic areas and the UK CHMI study reported previously[24,25] demonstrated an excellent safety profile combined with induction of potent cellular immune responses assessed by *ex vivo* IFN-γ ELISPOT. Here, we present additional analyses of cellular immunogenicity across all three groups (*i.e.*, Kenyan, Gambian, and UK adults) using both flow cytometry and IFN-γ ELISPOT on freshly isolated PBMC, as well as determination of humoral immunity by IgG enzyme-linked immunosorbent assay (ELISA) titer. Using this combination of simian adenovirus priming and poxvirus boosting vaccination, we demonstrate translation of the potent immunogenicity of vectored vaccines from malaria naive to semi-immune populations in Kenya and The Gambia.

**Table 1 Geometric mean IFN-γ ELISPOT responses by trial site, time point, and strain of *Plasmodium falciparum* used for TRAP peptide pool**

| Trial | Time point | TRAP strain | Geomean SFC | 95% CI | *P* value |
|---|---|---|---|---|---|
| Gambia + Kenya | 0 | T9/96 | 50 | 38–65 | 0.5 |
| UK | 0 | T9/96 | 46 | 33–65 | |
| Gambia + Kenya | 0 | 3D7 | 48 | 36–62 | 0.25 |
| UK | 0 | 3D7 | 61 | 36–103 | |
| The Gambia | 14 | T9/96 | 172 | 111–265 | <0.0001 |
| Kenya | 14 | T9/96 | 130 | 63–269 | 0.06 |
| UK | 14 | T9/96 | 242 | 122–480 | nt |
| The Gambia | 14 | 3D7 | 137 | 29–211 | <0.0001 |
| Kenya | 14 | 3D7 | 91 | 42–197 | 0.2 |
| UK | 14 | 3D7 | 194 | 94–402 | nt |
| The Gambia | 63 | T9/96 | 1015 | 765–1,347 | <0.0001 |
| Kenya | 63 | T9/96 | 467 | 247–885 | 0.006 |
| UK | 63 | T9/96 | 1524 | 728–3,194 | nt |
| The Gambia | 63 | 3D7 | 901 | 665–1,123 | <0.0001 |
| Kenya | 63 | 3D7 | 422 | 214–833 | 0.0005 |
| UK | 63 | 3D7 | 1009 | 473–2,150 | nt |
| The Gambia | 308 | T9/96 | 112 | 79–159 | 0.0004 |
| Kenya | 300 | T9/96 | 92 | 48–175 | 0.2 |
| The Gambia | 308 | 3D7 | 82 | 58–116 | 0.007 |
| Kenya | 300 | 3D7 | 82 | 38–177 | 0.2 |

*P* values are for Wilcoxon-matched-pairs signed rank test (two-tailed), comparing responses to postvaccination to those at time point 0. Responses in UK vaccinees at the final time point were not comparable as CHMI was performed at D77.
CHMI, controlled human malaria infection; CI, confidence interval; ELISPOT, enzyme-linked immunospot; IFN, interferon; nt, not tested; TRAP, thrombospondin-related adhesive protein.

## RESULTS

### High-level immunogenicity following prime-boost vaccination in semi-immune adults

Prevaccination responses to both strains of the TRAP antigen were compared using the *ex vivo* IFN-γ ELISPOT assay, between the African trial sites and a previous UK trial with the same vectors to assess pre-existing immunity to the candidate vaccine antigen.[24] No significant differences to either the T9/96 or 3D7 strain of *P. falciparum* (**Figure 1a** and **Table 1**) were observed between volunteers in the malaria-endemic regions where the field trials were performed and malaria-naive volunteers enrolled in a phase 2a controlled human malaria infection CHMI study of the same viral vectors in the United Kingdom.[24]

Priming vaccination with ChAd63 ME-TRAP induced modest T-cell responses after 14 days to peptides of the T9/96 strain of the TRAP antigen and these were slightly higher in Gambian than Kenyan vaccinees (group geometric means, 95% confidence intervals (CI) and *P* values for increases in ELISPOT response are shown in **Table 1**). Responses to the heterologous 3D7 strain of TRAP were significantly lower than those to the T9/96 vaccine strain in both trials at this time point (**Figure 1b**). Responses did not differ significantly to those in malaria naive adults.

Boosting with MVA ME-TRAP significantly increased the magnitude of IFN-γ ELISPOT responses to T9/96 in both trials, peaking at 1015 SFC/$10^6$ PBMC, (CI: 765–1,347) in the Gambian trial ($P < 0.0001$ compared to D14 responses) and 467 SFC/$10^6$ PBMC, (CI: 247–885; $P = 0.0064$) in Kenya. Peak responses in Gambians were higher than those in Kenyans ($P = 0.04$). When responses in vaccinees receiving the highest dose of ChAd63 ($5 \times 10^{10}$ viral particles (vp)) were combined across both trials, immunity to the T9/96 strain peaked at 1254 SFC/$10^6$ PBMC (CI: 912–1,724). Immunity to the 3D7 strain of TRAP also significantly increased after MVA vaccination to 901 SFC/$10^6$ PBMC in the Gambian (CI: 665–1,123; $P < 0.0001$) and 422 SFC/$10^6$ PBMC in Kenyan recipients (CI: 214–833; $P = 0.0005$; **Figure 1c**). For both strains, peak responses were lower in semi-immune adults than naive adults at the same time point, notably in Kenyans where the response was significantly lower ($P < 0.01$, Kruskall-Wallis test with Dunn's test for multiple comparisons, **Table 1**)

Immunogenicity was reassessed 8 months after MVA vaccination and plateaued at a level significantly higher than measured at baseline in Gambian participants. Responses in Kenyan volunteers at the same time point were only slightly higher than those detected prior to ChAd63 vaccination (**Figure 1d**).

Responses to the ME string of the ME-TRAP vaccine construct were substantially lower than to the TRAP antigen component.

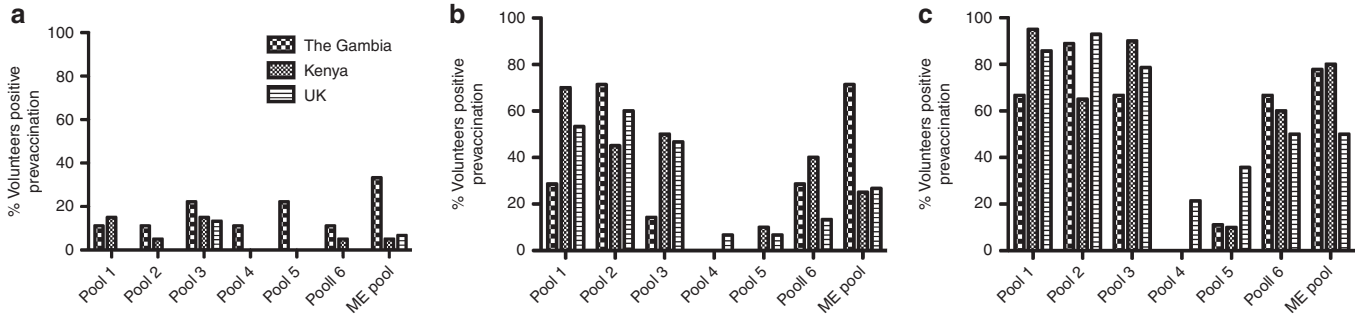


**Figure 2 Percentage of vaccinees responding to each peptide pool.** (**a**) prevaccination, (**b**) day 14 postprime with ChAd63 ME-TRAP, (**c**) 7 days postboost with MVA ME-TRAP. TRAP, thrombospondin-related adhesive protein.

In The Gambia, responses peaked at 94 SFC/$10^6$ PBMC (CI: 66–132), **Figure 1e** and 105 SFC/$10^6$ PBMC (CI: 51–215) in Kenya, **Figure 1f**. These responses did not differ significantly from those observed in malaria-naive volunteers in the United Kingdom at the same time point (**Figure 1g**).

## Vaccination induces broad responses to the TRAP antigen

At every time point studied, responses to the TRAP antigen were focused toward the first three peptide pools representing the *N*-terminal region, corresponding to amino acids (aa) 1-300, and the last peptide pool (aa 501-557) covering the *C*-terminal end. Responses to the central region (aa 300-500) of the antigen were rarely detected. The hierarchy of immunodominance for responses to the peptide pools was similar between malaria-naive and malaria-exposed vaccinees at each time point (**Figure 2a–c**). After each vaccination, the breadth of the immune response increased, as determined by the median number of peptide pools recognized, from 1 pool after ChAd63 vaccination in Kenyan participants to 3 after MVA vaccination. In Gambian participants, the breadth of response increased from 2 pools after ChAd63 to 3 at 1-week post-MVA and 4 pools at 5 weeks post-MVA.

## Prime-boost immunization induces significant increases in IgG responses in both African trials

Humoral immunity to TRAP before and after vaccination was assessed by IgG ELISA using a protein of the 3D7 strain of TRAP. Pre-existing anti-TRAP IgG responses were compared between malaria-naive and exposed volunteers. Titers in the UK volunteers were significantly lower than those in both Gambian and Kenyan vaccinees ($P < 0.001$, Kruskall-Wallis test with Dunn's multiple comparison posttest, **Figure 3a**).

There was no significant difference in antibody titer to TRAP between groups receiving low- or high-dose ChAd63 ME-TRAP at any time point in either trial ($P > 0.05$, two-tailed Mann–Whitney test, data not shown), and so, statistical analysis of increases was performed on combined data. Kinetics of antibody induction are shown in **Figure 3b**. Increases in antibody titers across both groups were significant after priming and boosting, ($P < 0.001$, Kruskall-Wallis test with Dunn's multiple comparison posttest comparing responses at D14 (Gambians only), D63, and D90 to baseline responses at D0). Peak responses at day 63 were 238 (CI: 159–358) ELISA units (EU) and 295 EU (CI: 235–370) in Gambian and Kenyan recipients respectively, and there was no significant difference in antibody response between sites ($P = 0.3$, two-tailed Mann–Whitney test).

## Antibodies to the adenovirus vector do not inhibit responses to the vaccine antigen

Pre-existing immunity to ChAd63 was generally low in both trials, with geometric mean titer of 192 (CI: 147–251) in Gambians and 214 (CI: 104–442) in Kenyans (**Figure 3c**). Fourteen days after immunization with ChAd63 ME-TRAP, titers increased significantly (The Gambia 1,157, CI: 710–1,890; Kenya 1,255, CI: 696–2,264; $P < 0.0001$ using two-tailed *t*-test on log transformed data comparing pre- and postvaccination titers for each trial), and there was a strong positive correlation between anti-ChAd63 neutralizing antibody titers pre- and postvaccination (Spearman's $r = 0.63$, $P < 0.0001$, **Figure 3d**). There was a weak negative correlation between pre-existing humoral immunity to ChAd63 and T-cell responses to the vaccine at day 14 (Spearman's $r = -0.3$, $P = 0.03$, **Figure 3e**), however this association disappeared after boosting with MVA (Spearman's $r = -0.2$, $P = 0.3$, **Figure 3f**), suggesting that boosting of pre-existing immunity to the vector by vaccination was not associated with inhibition of cellular immunity to the vaccine. There was a trend toward a weak positive relationship between pre-existing cellular immunity to ME-TRAP and the response to ChAd63-ME-TRAP (Spearman's $r = 0.3$, $P = 0.07$, **Figure 3g**). After boosting with MVA, a highly significant relationship between T-cell responses measured postprime and postboost was apparent (Spearman's $r = 0.6$, $P < 0.000\ 1$, **Figure 3h**).

## Dose of ChAd63 ME-TRAP and route of administration of MVA ME-TRAP do not affect immunogenicity

Cytokine secretion by $CD4^+$ and $CD8^+$ T cells was assessed by flow cytometry with intracellular cytokine staining using a single pool of peptides. Prior to vaccination, frequencies of cytokine-secreting T cells were low, with approximately equal distribution of cytokine production between $CD4^+$ and $CD8^+$ T cells in both trials (**Supplementary Figure S2a**). In Kenyan vaccinees, cytokine production was compared between volunteers vaccinated with MVA ME-TRAP via the intradermal (i.d.) and intramuscular (i.m.) routes. Prior to boosting with MVA, there were no significant differences in cytokine production between groups after correction for multiple comparisons for vaccinees primed with either

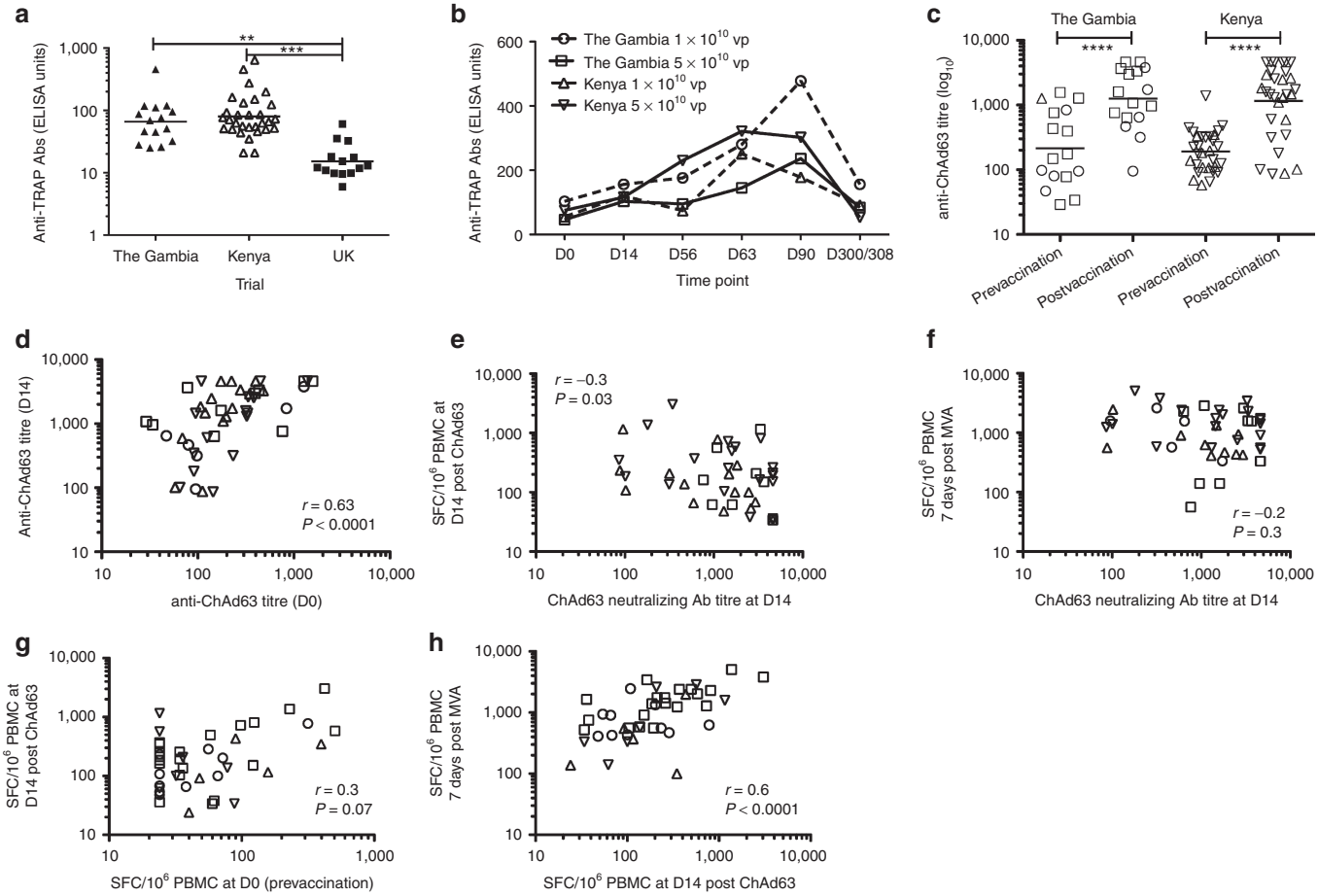


**Figure 3 Antibody responses and effect of pre-existing immunity on responses to vaccination.** (**a**) Prevaccination anti-thrombospondin-related adhesive protein (TRAP) IgG responses, $P < 0.001$ for UK versus Kenya, $P < 0.01$ for UK versus The Gambia analyzed using Kruskal-Wallis with Dunn's multiple comparison test. Line represents geometric mean. (**b**) Geometric means of log endpoint titers for IgG ELISA against 3D7 TRAP stratified by dose of ChAd63. Dashed line represents $1 \times 10^{10}$ viral particles (vp) and solid line $5 \times 10^{10}$ vp ChAd63 ME-TRAP, $P < 0.001$, Kruskal-Wallis test with Dunn's multiple comparison posttest comparing responses at D14, D63, and D90 to baseline responses at D0. (**c**) Neutralizing antibody titers to the ChAd63 vector pre- and postimmunization at 14 days for African trials, ****$P < 0.0001$, paired two-tailed *t*-test. (**d**) Correlation between pre- and postimmunization neutralizing antibody titers. (**e**) Lack of association between cellular immune response to TRAP and antibody response to the ChAd63 vector at 14 days postimmunization. (**f**) Postprime and postboost immunization neutralizing antibody titers. (**g**) Correlation between pre-existing immunity to the ME-TRAP vaccine and responses at 14 days postvaccination with ChAd63. (**h**) Relationship between magnitude of response to priming vaccination at day 14 post-ChAd63 and boosting vaccination at day 63 (7 days post-MVA). Spearman's two-tailed correlation coefficients shown with *P* value. Upwards pointing triangles represent vaccinees primed with $1 \times 10^{10}$ vp ChAd63 ME-TRAP in Kenya, downward pointing triangles $5 \times 10^{10}$ vp in Kenya, circles $1 \times 10^{10}$ vp ChAd63 ME-TRAP in The Gambia, and squares $5 \times 10^{10}$ vp ChAd63 ME-TRAP in The Gambia.

low ($1 \times 10^{10}$ vp, **Supplementary Figure S2b**) or high ($5 \times 10^{10}$ vp, **Supplementary Figure S2c**) doses of ChAd63 ME-TRAP. After boosting, at day 63 (7 days post-MVA), no significant differences were detected for different routes of MVA administration in either of the dose groups (**Supplementary Figure S2d,e**), confirming the findings previously reported of the same responses assessed by IFN-γ ELISPOT. Therefore, subsequent analyses are presented with data for different routes of MVA administration combined.

In both African trials, the effect of priming dose of ChAd63 ME-TRAP on the magnitude of cytokine-secreting T-cell frequencies was assessed by flow cytometry. Irrespective of the dose of the priming vector used, IFN-γ was predominantly secreted by $CD8^+$ T cells (pie-charts, **Figure 4a,b**). Secretion of IFN-γ, IL-2, and TNF-α could be detected from both $CD4^+$ and $CD8^+$ T cells at day 14, although responses tended to be higher in Kenyans. Seven days after boosting with MVA, IFN-γ secretion was more equally distributed between $CD4^+$ and $CD8^+$ T cells, particularly among vaccinees that received the higher dose of ChAd63 (pie-charts, **Figure 4c,d**).

No significant differences in magnitude of cytokine secreting T-cell populations were observed for different doses of ChAd63 within either trial; therefore, statistical analyses use data combined for both priming doses. At each trial site, frequencies of cytokine-secreting cells increased after each vaccination (**Table 2** and scatter plots, **Figure 4c,d**). Within individual trial sites, increases were not statistically significant, however when data across trials were combined, increases in the magnitude of $CD4^+$ IFN-$\gamma^+$ and $CD4^+$ TNF$\alpha^+$ populations reached significance ($P = 0.007$ and $P = 0.009$, Kruskall-Wallis test with Dunn's multiple comparison) and the increase in $CD4^+$ IL2-secreting

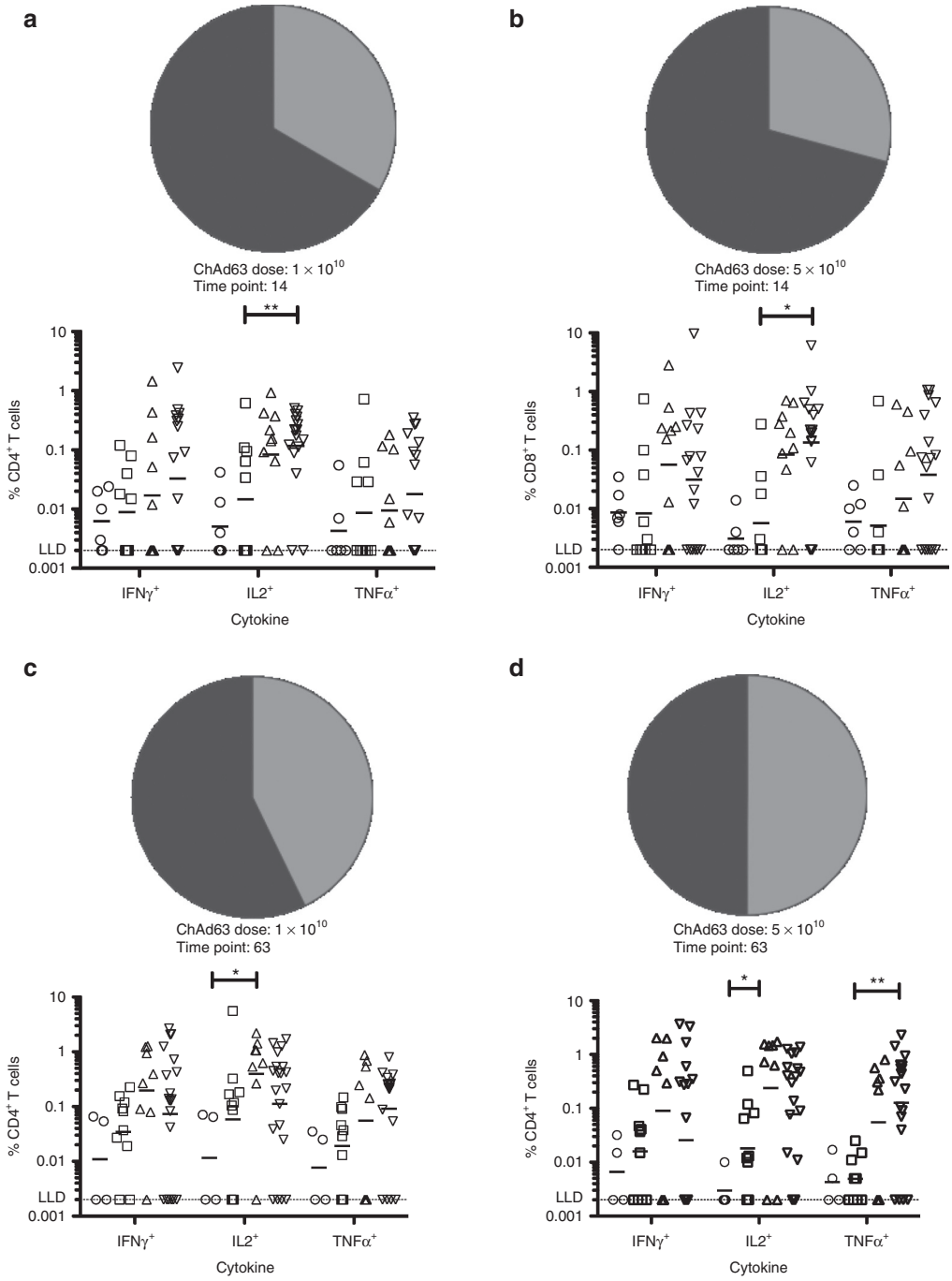


**Figure 4 Responses to prime-boost immunization with ChAd63 and MVA ME-TRAP by ICS.** Pie charts show proportion of IFN-γ secreted from $CD4^+$ (light grey) and $CD8^+$ (dark grey) as a proportion of total IFN-γ production. Scatter plots show individual responses to thrombospondin-related adhesive protein (TRAP) peptides from peripheral blood mononuclear cells stratified by trial and dose of priming immunization. Bars denote geometric mean. Cytokine frequencies from $CD4^+$ (**a**) and $CD8^+$ (**b**) T cells 14 days after priming immunization with ChAd63 ME-TRAP. **c** and **d** display cytokine secretion from $CD4^+$ (**c**) and $CD8^+$ (**d**) T cells after boosting with MVA ME-TRAP. Frequencies were compared with the Kruskall-Wallis test with Dunn's multiple comparison posttest, $^*P < 0.05$, $^{**}P < 0.01$. Upwards pointing triangles represent vaccinees primed with $1 \times 10^{10}$ viral particles (vp) ChAd63 ME-TRAP in Kenya, downward pointing triangles $5 \times 10^{10}$ vp in Kenya, circles $1 \times 10^{10}$ vp ChAd63 ME-TRAP in The Gambia, and squares $5 \times 10^{10}$ vp ChAd63 ME-TRAP in The Gambia. ICS, flow cytometry with intracellular cytokine staining; LLD, lower limit of detection of the assay.

cells showed a trend toward an increase after vaccination ($P = 0.07$). Increases in $CD8^+$ cytokine-secreting T-cell populations were not significant as frequencies of these populations were low. Analysis of polyfunctionality showed that a mixture of cell phenotypes was induced in all volunteers with single-, double-, and triple-cytokine secreting cells detected in both the $CD4^+$ and $CD8^+$ population (Figure 5a,b). $CD8^+$ T cells secreting IFN-γ, but not IL-2 or TNF-α (mono-functional), which have been previously shown to associate with protection against CHMI with this vaccine regimen were detected from vaccinees

**Table 2 Geometric mean frequencies of cytokine-secreting T cells by trial site and time point**

| T-cell population/ time point | Gambian vaccinees: geomean (95% CI) | Kenyan vaccinees: geomean (95% CI) | *P* value (sites combined) |
|---|---|---|---|
| $CD4^+$ T cells | | | |
| IFN-$\gamma^+$ | | | |
| d0 (pre-vacc) | 0.005 (0.002–0.01) | 0.02 (0.005–0.07) | 0.007 |
| d14 (post-ChAd63) | 0.008 (0.003–0.02) | 0.04 (0.01–0.1) | |
| d63 (7 days post-MVA) | 0.02 (0.008–0.07) | 0.1 (0.03–0.3) | |
| IL-$2^+$ | | | |
| d0 (pre-vacc) | 0.03 (0.009–0.09) | 0.05 (0.01–0.2) | 0.07 |
| d14 (post-ChAd63) | 0.01 (0.003–0.04) | 0.1 (0.04–0.2) | |
| d63 (7 days post-MVA) | 0.04 (0.007–0.2) | 0.2 (0.07–0.7) | |
| TNF$\alpha^+$ | | | |
| d0 (pre-vacc) | 0.009 (0.003–0.03) | 0.04 (0.01–0.2) | 0.009 |
| d14 (post-ChAd63) | 0.007 (0.002–0.02) | 0.01 (0.005–0.04) | |
| d63 (7 days post-MVA) | 0.01 (0.005–0.04) | 0.08 (0.03–0.2) | |
| $CD8^+$ T cells | | | |
| IFN-$\gamma^+$ | | | |
| d0 (pre-vacc) | 0.006 (0.002–0.02) | 0.03 (0.008–0.1) | ns |
| d14 (post-ChAd63) | 0.01 (0.003–0.03) | 0.06 (0.02–0.2) | |
| d63 (7 days post-MVA) | 0.01 (0.004–0.04) | 0.04 (0.009–0.2) | |
| IL-$2^+$ | | | |
| d0 (pre-vacc) | 0.009 (0.003–0.02) | 0.07 (0.02–0.3) | ns |
| d14 (post-ChAd63) | 0.005 (0.002–0.01) | 0.1 (0.05–0.3) | |
| d63 (7 days post-MVA) | 0.01 (0.004–0.04) | 0.2 (0.04–0.5) | |
| TNF-$\alpha^+$ | | | |
| d0 (pre-vacc) | 0.01 (0.003–0.03) | 0.1 (0.03–0.4) | ns |
| d14 (post-ChAd63) | 0.006 (0.002–0.02) | 0.03 (0.008–0.08) | |
| d63 (7 days post-MVA) | 0.004 (0.003–0.007) | 0.1 (0.03–0.3) | |

*P* values are for Kruskall Wallis test with Dunn's multiple comparison posttest comparing responses postvaccination to those at time point 0.
CI, confidence interval; IFN, interferon; IL, interleukin; MVA, modified vaccinia Ankara; ns, not statistically significant; TNF, tumor necrosis factor.

in both trials, however the frequency in volunteers from the trial in Kenya was significantly higher than that in The Gambia or the United Kingdom ($P = 0.008$, Kruskall-Wallis with Dunn's multiple comparison correction, **Figure 6**).

## DISCUSSION

Prime-boost vaccination with ChAd63 and MVA ME-TRAP has demonstrated excellent immunogenicity in these two phase 1b trials in healthy adults in regions of low-to-moderate malaria transmission. Despite presumably repeated exposure to malaria, pre-existing baseline T-cell responses to the TRAP antigen measured by *ex vivo* ELISPOT did not differ between malaria-naive and exposed volunteers, suggesting that TRAP is not a major target for naturally acquired cellular immunity. Similarly, after priming and boosting immunizations, T-cell responses to the vaccine antigen were of the same magnitude as those in UK malaria-naive vaccinees, suggesting that vaccination does not boost T-cell responses primed by natural exposure, despite the evidence of central memory responses to TRAP in Kenyan adults.[19]

The T9/96 and 3D7 strains of *P. falciparum* represent the breadth of genetic diversity of the TRAP antigen with 6.1% amino acid variation.[26] Despite this, responses to the heterologous 3D7 strain (used for CHMI studies) were of a similar order of magnitude in both cohorts suggesting that this vaccine will not be affected substantially by antigenic polymorphism, as T cells induced by vaccination with the T9/96 strain were highly cross-reactive. Responses were detected to pools representing 46 of the 56 peptides spanning the TRAP protein (82%), demonstrating the high number of potential epitopes within this antigen. The breadth of the immune response induced by vaccination was also large and indicated that a number of epitopes over the length of the antigen were recognized. Given the potential diversity of HLA types in populations that might be targeted with a malaria vaccine, the finding of potentially highly promiscuous epitopes is encouraging.

More than a decade ago, the approach of combining a DNA priming vaccination with MVA boosting using the same ME-TRAP construct induced very modest T-cell responses by *ex vivo* ELISPOT (GM 69.8 SFC/$10^6$ PBMC) that failed to reduce the rate of malaria infection in Gambian men,[27,28] most likely as a result of insufficient immunogenicity. Previous trials of prime-boost viral vectored vaccines with this antigen that employed two pox viruses (albeit different viruses) as both priming and boosting vectors failed to elicit sufficiently potent immunity to mediate protection in volunteers in coastal Kenya or The Gambia.[18–20] Although the immunogenicity induced by the fowlpox-MVA ME-TRAP vaccination regime was more immunogenic than DNA-MVA, the peak response was still modest at 185 SFC/$10^6$ PBMC. After a double prime with fowlpox ME-TRAP, peak responses reached 389 SFC/$10^6$ PBMC, however this was also insufficient to induce protection against febrile malaria among young children in Kenya. In adults, immunogenicity in both settings was lower than in malaria-naive adults, most likely due to immunosuppression caused by malaria. Data from Kenyan children showed reduced T-cell responses after both vaccination with poxviruses, and natural exposure was associated with parasitaemia and clinical malaria.[29]

Here, we show that replacement of the fowlpox or DNA priming vectors with the chimpanzee adenovirus vector has both increased the magnitude of the peak response fourfold to 1254 SFC per million PBMC and resulted in increased induction of $CD8^+$ T-cell-mediated immunity—a prerequisite for an effective pre-erythrocytic vaccine.[30] This suggests that adenoviral vectors may be more robust to the immunosuppressive effects of malaria than poxvirus vectors, a potentially useful characteristic for vaccines against other diseases in malaria-endemic regions. In addition, responses were maintained above baseline in the Kenyan vaccinees for 8 months, demonstrating useful durability of effector memory type cellular immunity and potentially greater longitudinal central memory. Vaccination with ChAd63 and MVA ME-TRAP also induced modest, but significant increases in TRAP IgG levels, despite a higher level of pre-existing humoral immunity than in malaria-naive vaccinees, suggesting that prior immunity to TRAP does not inhibit development of vaccine-induced antibodies.

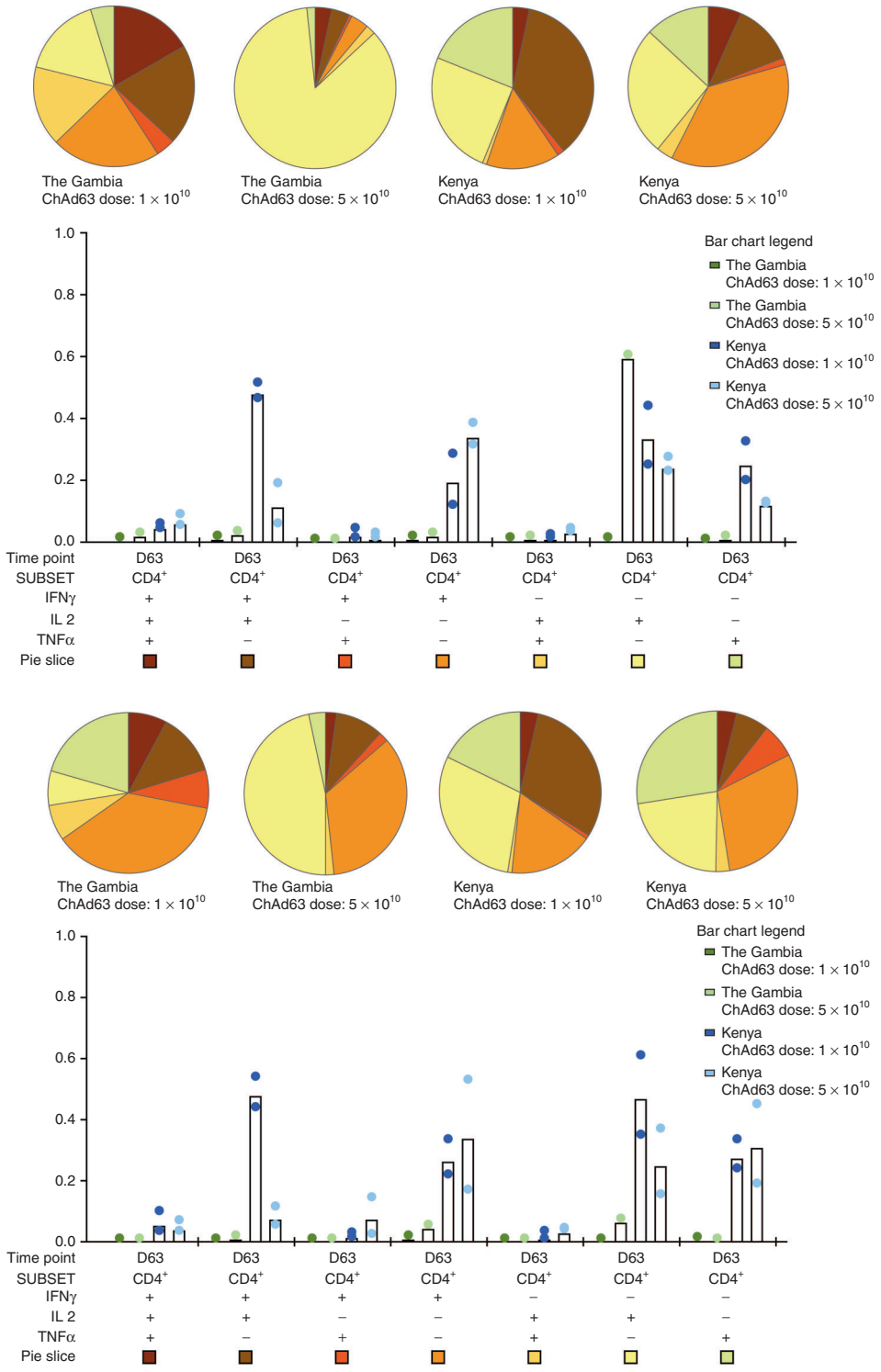


**Figure 5 Polyfunctionality of peak T-cell responses at day 63.** Analysis of cytokine secretion from $CD4^+$ and $CD8^+$ T cells was performed using a Boolean gating strategy in SPICE. Pie charts represent the proportion of the immune response represented by each combination of cytokines shown in the bar chart. Bar charts show the frequency of each population with bars representing median responses. (**a**) $CD4^+$ responses. (**b**) $CD8^+$ responses. SPICE, simplified presentation of incredibly complex evaluations.

Contrary to prevailing theory, pre-existing immunity to this adenoviral vector did not inhibit the subsequent immunogenicity of the vaccine, in fact a trend toward a weak positive association was detected. Our group has previously demonstrated a much stronger positive association in UK volunteers, however the finding reported here in regions of increased prevalence of neutralizing antibodies to the adenoviral vector is pertinent for the potential deployment of this vaccine in an endemic population and similar levels of seroprevalence to this vector have recently been reported in Burkina Faso.[31] Given the strong correlation

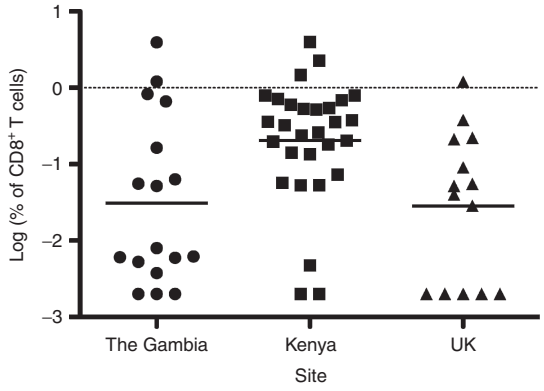


**Figure 6 Frequency of monofunctional CD8$^+$ T cells (CD8$^+$ T cells secreting IFN-γ, but not IL-2 or TNF-α) was significantly higher in Kenyan than Gambian or UK vaccinees.** Frequencies were compared with the Kruskall-Wallis test with Dunn's multiple comparison posttest, *$P < 0.05$. Line shows geometric mean.

between responses after priming and boosting, the magnitude of the peak response is clearly related to the magnitude of the response to ChAd63 ME-TRAP, rather than any humoral response to the adenoviral vector.

The dose of ChAd63 used for priming did not significantly affect the immunogenicity of the vaccine by any of the measures we studied, in agreement with the first UK phase 1 dose-escalation study of the ChAd63 ME-TRAP priming immunization.[21] Furthermore, administration of the boosting MVA ME-TRAP vector by either the i.m. or i.d. routes had no effect on responses to the TRAP insert. Given that i.m. immunization is easier to perform with reduced local reactogenicity,[25] future trials of the MVA ME-TRAP vaccine will employ this route of administration.

Phenotyping of vaccine-induced T-cell subtypes by flow cytometry with intracellular cytokine staining revealed production of IFN-γ, IL-2, and TNF-α from CD4$^+$ and CD8$^+$ T cells in all combinations. In a previously reported study of the efficacy of ChAd63 and MVA ME-TRAP in the United Kingdom, we showed 21% sterile efficacy increasing to more than 50% efficacy when including delay to detectable parasitaemia.[24] We then demonstrated a correlation between efficacy and frequency of monofunctional CD8$^+$ T cells secreting IFN-γ, a finding also observed in a nonhuman primate model of sporozoite-induced protection.[32] In this study, the frequency of this protective CD8$^+$ T-cell population was significantly higher in Kenyan vaccinees than in the UK study, suggesting that this vaccine may be more protective in this population, particularly given that the infectious inoculum during natural exposure may well be lower than in the CHMI model of sporozoite challenge.[33]

Although practical limitations prevented flow cytometric analysis of the memory phenotype of the vaccine-induced T-cell response, previous studies in mice using the same vaccine regimen clearly identified CD8$^+$ T effector memory T cells as essential for protection against the pre-erythrocytic stage of malaria.[12] In humans, the exact nature of T-cell phenotype best suited to mediating durable protective immunity is not clear, with different models proposing both effector and central memory T cell as more important.[34,35] Future studies of vaccine efficacy could address these conflicting positions, although markers for discrimination of effector and memory populations in human T cells are complex due to the heterogeneous nature of the phenotypes.[36]

The initial report of these two phase 1 clinical trials demonstrated excellent safety with very acceptable reactogenicity profiles for both ChAd63 and MVA ME-TRAP.[25] In this report, we have detailed the very potent cellular immunogenicity of this vaccine for two increasing doses of the ChAd63 priming vector and two routes of administration for the MVA vector. This regimen elicits a broad CD4$^+$ and CD8$^+$ T-cell-mediated immune response including a phenotype shown to induce protective efficacy against sporozoite challenge. The combination of an acceptable safety profile and impressive immunogenicity provides a clear rationale for phase 1 age de-escalation studies in children in semi-immune populations and phase 2b efficacy studies in adults in endemic regions. Studies of both such designs are underway. The data presented here also highlight the potential utility of these viral vectors as vaccines for other diseases where induction of high levels of T-cell immunity is helpful or necessary for protection.

## MATERIALS AND METHODS

The objectives, setting, participant demographics, clinical study design, and methods for vaccine production used in these clinical trials have been described in detail elsewhere.[25] Brief details are repeated here.

***Study settings and design.*** The first trial was conducted at the Medical Research Council field site located within Sukuta Health Centre in Kombo North district of The Gambia, West Africa. Sukuta village has an estimated population of 17,000 (2003 census). The climate is typical of sub-Saharan Africa with a long dry season lasting from December to June followed by a relatively short rainy season from July to November when the majority of *P. falciparum* malaria transmission occurs.[37]

The second trial was conducted in Vipingo, Kilifi County, Kenya, East Africa. Participants were recruited from the Rea Vipingo Sisal Plantation Estates in Kilifi, which has over 1,000 employees and a land area of 3,950 hectares. In Kilifi, there are two seasons of high transmission of *P. falciparum* malaria coinciding with the long monsoon rains (April to June) and the short rains (October to December).[38]

The third trial was conducted in Oxford, UK, and local malaria-naive volunteers were recruited.

We conducted two phase 1b open-label malaria vaccine trials. Both trials evaluated low- ($1\times10^{10}$ vp) and high-dose ($5\times10^{10}$ vp) ChAd63 ME-TRAP. The trial in Kenya also compared i.m. and i.d. routes of administration of $2\times10^{8}$ p.f.u. MVA ME-TRAP. In The Gambia, eligible participants were allocated to receive either ChAd63 ME-TRAP $1\times10^{10}$ vp (group 1; $n = 6$) or ChAd63 ME-TRAP $5\times10^{10}$ vp (group 2; $n = 10$) administered i.m. in the deltoid. All participants were subsequently vaccinated in the opposite arm 56 days later with $2\times10^{8}$ plaque forming units (p.f.u.) MVA ME-TRAP administered i.m.. In the Kenyan trial, eligible participants were allocated to receive either ChAd63 ME-TRAP $5\times10^{9}$ vp (group 1; $n = 10$) or ChAd63 ME-TRAP $5\times10^{10}$ vp (group 2; $n = 20$) administered i.m. in the deltoid. All participants were subsequently vaccinated in the opposite arm 56 days later with $2\times10^{8}$ p.f.u. MVA ME-TRAP. Participants in each group were randomized 1:1 to receive MVA ME-TRAP administered i.m. or i.d. Blood samples for immunology were collected on day 0 (day of ChAd63 ME-TRAP vaccination), day 14, day 28, day 56 (day of MVA ME-TRAP vaccination), day 63, day 90, and day 300 (The Gambia) or day 308 (Kenya).

The UK trial was a phase 2a sporozoite challenge study (CHMI) with volunteers receiving ChAd63 ME-TRAP $5\times10^{10}$ vp administered i.m. followed by of $2\times10^{8}$ p.f.u. MVA ME-TRAP administered i.d. 56 days later.[24] Data shown here are from blood samples collected for immunology on day 0, 14, 28, 56, and 63; no data are shown post-CHMI. Some data were reanalyzed for comparability with methods employed in this study.

In particular, the IgG antibody data shown here were performed using a standardized anti-TRAP ELISA rather than the end-point titer assay described in the previous publication, however agreement between the two methods was excellent and data correlated extremely well (Spearman's $r = 0.9$, two-tailed $P < 0.0001$).

***Ethical and regulatory approval.*** The clinical trial protocols and associated documents were approved by Gambia Government/Medical Research Council Joint Ethics Committee and The Kenya Medical Research Institute National Ethics Committee, respectively. Documents for all clinical trials were reviewed and approved by the Oxford Tropical Research Ethics Committee. Regulatory approval was given by the Medicines Board of The Gambia, The Pharmacy and Poisons Board of Kenya, and the Medicines and Healthcare products Regulatory Agency, respectively. All participants gave documented informed consent prior to any study procedure being undertaken. The study was conducted according to the principles of the Declaration of Helsinki (2008) and the International Conference on Harmonization Good Clinical Practice guidelines. An independent Data and Safety Monitoring Board and local safety monitors provided safety oversight and Good Clinical Practice compliance was independently monitored by an external organization at both trial sites (Appledown Clinical Research, Great Missenden, UK). Trials were registered with Pan African Clinical Trial Registry (Ref: PACT ATMR 2010020001771828 & PACTR 201008000221638); http://www.pactr.org/ and ClinicalTrials.gov (Ref: NCT01373879, NCT01379430 & NCT00890760); http://clinicaltrials.gov/ct2/home.

***Standardization of immunological methods.*** Staff from the Kenyan and Gambian immunology laboratories visited The Jenner Institute for training on immunology protocols that were standardized across all three sites. Staff from the Jenner Institute visited both African institutes to help with sample processing and equipment optimization. For cellular immunology, reagents were provided from The Jenner Institute after batch testing and the same batch of peptides were used in all studies. ELISAs and neutralizing antibody assays on samples from The Gambia and UK studies were performed at the Jenner Institute and at KEMRI for the Kenyan study using reagents supplied by the Jenner Institute. Data analysis was largely performed independently at each site with quality control performed by a single senior scientist at the Jenner Institute. All assays of cellular immunity reported here were performed on freshly isolated PBMC.

**Ex vivo *ELISPOT assays.*** *Ex vivo* (18-hour stimulation) ELISPOT assays were performed using Multiscreen IP ELISPOT plates (Merck Millipore, Billerica, MA), human IFN-γ SA-ALP antibody kits (Mabtech, Stockholm, Sweden), and BCIP NBT-plus chromogenic substrate (Moss, Pasadena, MD). Cells were cultured in RPMI (Sigma, Poole, UK) containing 10% heat-inactivated, sterile-filtered fetal calf serum, previously screened for low reactivity (Labtech International, Uckfield, UK). Antigens were tested in duplicate with 250,000 freshly isolated PBMC added to each well of the ELISPOT plate.[16] TRAP peptides were 20 amino acids in length, overlapping by 10 amino acids (Neopeptide, Cambridge, MA), assayed in 6 pools of 7–10 peptides at 10 μg/ml. Responses were averaged across duplicates, responses in unstimulated (negative control) wells were subtracted, and then responses in individual pools were summed for each strain of the TRAP antigen. ME responses were assayed in a single pool and peptide pool configurations are shown in **Supplementary Tables S1 and S2**. Staphylococcal enzyme B (SEB, 0.02μg/ml) and phytohemmagglutinin-L (10 μg/ml, Sigma) were used as a positive control. Plates were counted using an AID automated ELISPOT counter (AID Diagnostika GmbH, Strassberg, Germany; algorithm C), using identical settings for all plates, and counts were adjusted only to remove artifacts. Responses to the negative control were always less than 90 SFC per million PBMC and the median across both trials was 11 SFC per million PBMC. Pools were considered positive if the response was greater than 11 SFC per million PBMC and four times higher than the negative control for that assay. The lower limit of detection for the assay is 24 SFC per million PBMC for peptide pools and 4 SFC for individual pools.

***Flow cytometry.*** PBMC were separated by density centrifugation from heparinized whole blood and resuspended in polypropylene tubes at $1 \times 10^6$ PBMC per ml in RPMI containing 10% heat-inactivated, batch-tested, sterile-filtered fetal bovine serum, 1% L-glutamine, 1% penicillin/streptomycin, and 1 μg/ml each of anti-CD28 and anti-CD49d (BD Biosciences, Oxford, UK). Samples were stored at room temperature prior to processing, which was completed within 6 hours of venipuncture. Cell counts were performed using trypan blue staining and a microscope according to an established SOP in the laboratory. Cells were then stimulated overnight at 37 °C with 5% $CO_2$, either with a pool of 56 peptides at 2 μg/ml spanning the entire length of the TRAP protein from the T9/96 strain of *P. falciparum* comprising 20mers overlapping by 10 amino acids, SEB or an unstimulated control containing an equivalent concentration of DMSO to the TRAP peptide pool (0.3%). Brefeldin A (BD Biosciences) was after 2 hours into the incubation at 1 μg/ml. Cells were then washed in fluorescence-activated cell sorting buffer (phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.01% sodium azide, Sigma) and stained for viability with LIVE/DEAD aqua amine reactive dye (Life technologies, Carlsbad, CA) for 20 minutes at 4 °C in the dark, after which a cocktail of antibodies for surface marker staining was added and incubated for 30 minutes at 4 °C in the dark. Cells were washed again in fluorescence-activated cell sorting buffer, then permeabilized for 30 minutes with Cytofix/Cytoperm (BD Biosciences), and washed in permeabilization buffer (BD Biosciences). A cocktail of antibodies for intracellular staining was then added and incubated for 30 minutes at room temperature in the dark. Antibody cocktails are described in **Supplementary Table S3**. Cells were washed in permeabilization buffer and resuspended in PBS containing 1% paraformaldehyde, prior to acquisition on CyAn flow cytometers (Beckman Coulter, High Wycombe, UK) on the day of staining. Compensation control beads (Compbeads, BD Biosciences) were stained according to the manufacturers instructions using the same concentration of antibody used for cells to for compensation between parameters, and unstained cells were used to adjust forward and side scatter photo-multiplier tube voltages.

***Analysis of flow cytometry data.*** At least 50,000 live $CD3^+$ cells were analyzed per sample. Data were prepared and analyzed using FlowJo v8.8.6 (Treestar, Ashland, Oregon), Pestle v1.6 and Spice v5.05 (Mario Roederer, Vaccine Research Centre, NIAID, NIH). A hierarchical gating strategy was used as described in **Supplementary Figure S1**. Responses to peptide were determined after subtraction of the response in the unstimulated control for each sample. Pie charts were created using absolute measures with a threshold of 0.002%. For analyses of multiple cytokine function, all samples had >10,000 $CD4^+$ or $CD8^+$ T cells in the parent population. Samples where a response to the positive control of greater than 1% cytokine positive $CD4^+$ or $CD8^+$ T cells could not be detected were excluded from the analysis. The lower limit of detection for the assay was 0.002%, and a positive response was greater than two times the medium control for the corresponding sample.

***Antivector neutralizing antibody assay.*** One day prior to performing the assay, GripTite 293 cells (Life Technologies) were seeded in 96-well plates ($3 \times 10^4$ cells/well). Heat-inactivated test samples were diluted fourfold from 1:9 to 1:2,304 in 10% sterile-filtered fetal bovine serum in Dulbecco's modified Eagle's medium and incubated 1:1 with ChAd63 expressing the secreted alkaline phosphatase gene ($8 \times 10^7$ vp/ml) for 1 hour at 37 °C. Serum and virus were then added to 293 cells in a volume of 200 μl in duplicate for 1 hour, after which sample and virus were aspirated and replaced with fresh 10% fetal bovine serum Dulbecco's modified Eagle's medium. A virus-only control was included. After 22–26 hours at 37 °C, 50 μl of medium was assayed for SEAP activity using a Phospha-Light TROPIX phosphatase assay (Life Technologies) in black assay plates, and luminescence was measured after

45 minutes on a Varioskan Flash Luminometer (ThermoFisher, Waltham, MA). Antivector neutralization titers were defined as the dilution of serum showing 50% reduction in SEAP activity, based on observed % inhibition values relative to SEAP activity from virus alone. Antivector antibodies were measured in serum in The Gambia and plasma for Kenyans, according to sample availability and local clinical practice, however we have determined that these sample types are equivalent for this assay.

***Serum IgG antibody responses.*** Antibody responses were measured by anti-TRAP IgG sandwich ELISA. Nunc-Immuno 96 well plates were coated with 0.5 µg/ml of TRAP antigen in carbonate-bicarbonate coating buffer and left overnight at 4 °C. Plates were washed 6× with PBS-Tween (PBS/T), then blocked with 1% BSA in PBS/T for 1 hour at room temperature. Serum or plasma was diluted in PBS/T containing 0.2% BSA at concentrations of 1:100, 1:500, or 1:1,000, and added in triplicate. Samples from days 0 (ChAd63 vaccination), 28, 56 (MVA vaccination), 63, and 300 were analyzed. Plates were incubated at room temperature for 2 hours; then washed as before. A secondary antibody (goat anti-human whole IgG conjugated to alkaline phosphatase, Sigma) was added at a dilution of 1:1,000 in PBS/T 0.2% BSA for 1 hour at room temperature. After a final wash, plates were developed by adding 4-nitrophenyl phosphate in diethanolamine buffer (Pierce, Loughborough, UK).

A positive reference standard (made from pooled TRAP-positive serum) was used on each plate to give a standard curve. It was added in duplicate at an initial dilution of 1:100 (in PBS/T 0.2% BSA) and diluted twofold 10 times, starting with an arbitrary value of 20 antibody units. Four blank wells (zero antibody units) were also designated. The optical density values were then fitted to a 4 parameter standard curve using SOFTmax PRO software.[15] An internal control was included on every plate in triplicate made up from a 1:800 dilution (in PBS/T 0.2% BSA) of the positive standard. Optical density was read at 405 nm using an ELx800 microplate reader (Cole Parmer, London, UK). Test sera antibody units were calculated from their optical density values using the parameters estimated from the standard curve.

***Statistical analysis.*** Group data are geometric means unless otherwise stated with 95% CI. The matched pairs analysis excludes volunteers with missing data at any time point. A Kruskall-Wallis test was used to compare increases in T-cell frequencies in time courses with Dunn's multiple comparisons posttest used to compare response pre- and postvaccination. For statistical analyses, an $\alpha$-level of 0.05 was considered significant and all *P* values are two-tailed. All analyses were performed in GraphPad Prism, Mac version 5 (GraphPad, La Jolla, CA).

## SUPPLEMENTARY MATERIAL

**Figure S1.** Sample gating strategy for flow cytometry analysis.
**Figure S2.** Effect of route of MVA administration on responses to TRAP peptides by ICS, expressed as the percentage of cytokine-secreting $CD4^+$ or $CD8^+$ T cells.
**Table S1.** TRAP peptide pool format.
**Table S2.** ME peptide pool format.
**Table S3.** Reagents used for flow cytometry staining.

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