

Prime and Target Immunization Protects Against Liver-Stage Malaria in Mice

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Overline: Malaria

One Sentence Summary

A prime and target vaccine improves current regimens for liver stage malaria resulting in sterile protection in mouse disease models and appears safe for human use.

Abstract

Despite recent advances in treatment and vector control, malaria is still a leading cause of death, emphasizing the need for an effective vaccine. The malaria life-cycle can be subdivided into three stages: the invasion and growth within liver hepatocytes (pre-erythrocytic stage), the blood- (erythrocytic stage) and finally the sexual-stage (occurring within the mosquito vector). Antigen-specific CD8⁺ T-cells are effectively induced by heterologous prime-boost viral vector immunization and known to correlate with liver-stage protection. However, liver-stage malaria vaccines have struggled to generate and maintain the high numbers of *Plasmodium*-specific circulating T-cells necessary to confer sterile protection. Here, we describe an alternative “prime and target” vaccination strategy aimed specifically at inducing high numbers of tissue-resident memory T-cells present in the liver at the time of hepatic infection. This approach bypasses the need for very high numbers of circulating T-cells and markedly increases the efficacy of subunit immunization against liver-stage malaria using clinically relevant antigens and clinically tested viral vectors in murine challenge models. Translation to clinical use has begun, with encouraging results from a pilot safety and feasibility trial of intravenous chimpanzee adenovirus vaccination in humans. This work highlights the value of a prime-target approach for immunization against malaria and suggests that this strategy may represent a more general approach for prophylaxis or immunotherapy of other liver infections and diseases.

Introduction

Malaria, caused by *Plasmodium* species, is an important disease causing about 500,000 deaths annually, with particularly high morbidity and mortality in sub-Saharan Africa (1). With growing antimalarial drug and insecticide resistance, an effective vaccination regimen is needed urgently (2). A variety of different subunit vaccine strategies have been investigated: RTS,S/AS01, a protein-in-adjuvant formulation inducing antibodies against circumsporozoite protein (CSP), completed Phase-III field trials with partial efficacy, but is still years from licensure (3).

An alternative approach to induce protection against pre-erythrocytic malaria has been to generate cellular immunity directed against infected hepatocytes. Seminal studies in mice demonstrated the ability of Interferon γ (IFN γ)-producing CD8⁺ T-cells to protect against liver-stage malaria (4, 5). To this end, substantial effort has been invested in optimizing a viral vector heterologous prime-boost strategy (known to be particularly effective at inducing T-cell responses) able to generate high frequencies of antigen (Ag) specific CD8⁺ T-cells (6, 7). A leading approach comprises a chimpanzee adenovirus (ChAd) viral vector prime, subsequently boosted with Modified Vaccinia Ankara (MVA) expressing the same antigen (such as Thrombospondin-Related Adhesion Protein (TRAP)), resulting in high numbers of CD8⁺ T-cells in circulation with partial clinical efficacy in both malaria naïve and pre-exposed individuals (8-11). The induced CD8⁺ T-cell numbers correlate with efficacy in Controlled Human Malaria Infection (CHMI) challenges, suggesting that increased numbers of CD8⁺ T-cells would be required for higher protection.

Vaccines aimed at inducing cell-mediated immunity against malaria have been hindered by challenging spatio-temporal factors pertaining to parasite biology and liver microanatomy. Firstly, using only peripheral vaccination regimens, it is difficult to generate and maintain the high numbers of *Plasmodium*-specific T-cells necessary to confer protection (12). Secondly, it has been difficult to direct a robust cell-mediated immune response to the liver to enable parasite clearance in the short time window of liver-stage malaria (5-7 days for *P. falciparum* infections in humans, 2 days in *P. berghei* infections in mice) (13-16). In recent years, tissue-resident (T_{RM}) T-cells have been identified (17-21) and shown to act as sentinels against invading pathogens under certain settings (22, 23). Indeed, CXCR6⁺ CD8⁺ T-cells (enriched following radiation-attenuated

spz vaccination), when lacking CXCR6 expression, result in both reduced numbers of liver memory T-cells and protection during spz challenges (24, 25). Furthermore, intravenous (i.v.) but not intramuscular (i.m.), administration of irradiated-cryopreserved spz generated high-frequencies of hepatic T-cells in non-human primates (26).

T_{RM} have been generated experimentally by recruiting effector CD8⁺ T-cells into tissues by administration of monoclonal antibodies (mAb) fused to pathogenic epitopes (27), chemokines (28, 29), inflammatory agents (30), and inactivated pathogens (31). While this work was in progress, the use of peptide-antigen conjugated to mAb specific for splenic CD8⁺ dendritic cells, followed by i.v.-injection of recombinant adeno-associated virus vector (AAV), was shown to generate high frequencies of liver T_{RM}, mediating sterile protection against liver-stage malaria (32). Our approach here focused on identifying a highly translatable and deployable vaccine strategy that can first induce abundant circulating T-cells against full length Ag in diverse human populations, and subsequently increase liver CD8⁺ T-cells expressing T_{RM} markers by targeting the second dose of immunogen to the liver. Targeting CD8⁺ T-cells to the liver was achieved using either poly(lactic-co-glycolic acid) (PLGA)-protein loaded nanoparticles (Nps) or a recombinant viral vector. This approach generates durable liver Ag-specific CD8⁺ T-cells for up to 6-months and strikingly increases the efficacy of subunit immunization against liver-stage malaria using clinically relevant vaccines. Clinical development of this prime-target approach in humans has begun with a small Phase I trial, in which healthy volunteers have received i.v. vaccination of a ChAd-based vaccine with encouraging safety and immunogenicity data.

Results

Prime and target regimen results in sterile protection upon *P. berghei* challenge

In an effort to direct cell-mediated immune responses to the liver, we initially utilized nanoparticles. Consistent with previous data (33), following i.v.-administration PLGA-Np containing Ovalbumin (OVA) conjugated to Allophycocyanin (APC) (Np.APC-OVA) were found to be concentrated within hepatic tissue and taken up by Kupffer cells (fig. S1A, B; Movie S1). In contrast, i.m. injection of Np did not show liver-specific concentration (fig. S1B). Adoptively transferred naïve OVA-specific OTI, but not

naïve polyclonal, CD8⁺ T-cells clustered and increased CD69 expression at sites of Np.APC-OVA uptake by Kupffer cells, indicating that the associated OVA antigen had been processed and presented by MHC-I molecules (34) (fig. S1C).

To evaluate the ability of Np to promote localization of primed effector/memory CD8⁺ T-cells to the liver and mediate protection against liver-stage malaria, mice were primed with human adenovirus-5 expressing OVA (Ad.OVA-i.m.) and two weeks later Np.OVA were administered i.v. (Ad.OVA-i.m./Np.OVA-i.v.; fig. S2A). Protection was assessed by challenging vaccinated mice with two different transgenic (Tg) *P. berghei* sporozoites expressing OVA (OVA-spz) (35). Vaccine efficacy was determined as the time to reach the endpoint of 1% blood parasitemia. Strikingly, after Ad.OVA-i.m./Np.OVA-i.v. vaccination, 100% of mice displayed sterile protection in both challenge models, whereas Ad.OVA-i.m. or Ad.OVA-i.m./Np.OVA-i.m. failed to protect the animals (**Fig. 1A, B**; fig. S2B). Subsequent flow cytometry analysis showed that Ad.OVA-i.m./Np.OVA-i.v. immunization, but not Ad.OVA-i.m./Np.OVA-i.m. or Ad.OVA-i.m., produced a ten-fold increase in frequency and total number of OVA-specific (SIINFEKL-Pentamer (Pen⁺) binding) CD8⁺ T-cells in the liver, as well as a modest increase in CD4⁺ T-cells, with no changes observed in the spleen (**Fig. 1 C-H**, fig. S2C). In contrast, i.m. administration of Np (Ad.OVA-i.m./Np.OVA-i.m.) increased the Pen⁺ CD8⁺ T-cell frequency in the dLN, but not in the liver or spleen. These data suggest that concentrating the primed CD8⁺ T-cells in the liver, the site of initial malaria infection, may play an important role in mediating protection. To investigate whether a Np-i.v. boosting increased Ag-specific T-cells within the liver and/or in peripheral secondary lymphoid sites, we administered FTY720 to limit egress of activated T-cells from lymphoid sites (36), and pulsed-BrdU to track cell proliferation. These data suggested that both *in situ* proliferation in secondary lymphoid tissues and a migration of Pen⁺ CD8⁺ T-cells to the liver was occurring (fig. S3). Lastly, we attempted to improve immunogenicity of prime-target by conjugating Np to adjuvants previously shown to increase T-cell responses (37). However, addition of adjuvant (Resiquimod (R848) and Monophosphoryl Lipid A (MPL)) only marginally increased liver Ag-specific T-cells (fig. S4). Prime-target vaccination involving Np.OVA-i.v. alone generates particularly high numbers of Ag-specific CD8⁺ T-cells in the liver tissue and results in sterile protection from spz challenge.

CD8⁺ T-cells cluster around infected hepatocytes resulting in effector responses

To study the cellular mechanisms underlying this protective response, we conducted multi-parameter confocal imaging. Mice were immunized with either Ad.OVA-i.m./Np.OVA-i.v. or Ad.OVA-i.m. only and three weeks later challenged with OVA-spz or non-transgenic spz, with livers harvested 12-hours later. Livers from Ad.OVA-i.m./Np.OVA-i.v., but not Ad.OVA-i.m. animals, showed OVA-spz surrounded by large clusters of Kupffer cells (F4/80⁺) and polyclonal CD8⁺ T-cells producing IFN γ and Granzyme B (**Fig. 2A-C**; fig. S5A). Additionally, some of the clustered cells were Ki67⁺, indicating recent cell proliferation (38) (**Fig. 2B, D**). In contrast, mice given only Ad.OVA-i.m. showed no cell accumulation or effector staining around infected hepatocytes suggesting that at 12-hours post-infection few cells were actively recruited (**Fig. 2A, B**; fig. S5A). As previously observed in the liver (39-41), these T-cell clusters were only present in animals challenged with spz-OVA parasites while smaller size clusters were found in animals challenged with spz not expressing OVA. The low number of lymphocytes present around non-transgenic spz suggests that large cluster formation requires cognate antigen recognition. Immunizing animals with an irrelevant encapsulated protein (Np.BSA-i.v.) failed to confer protection against OVA-spz challenge (fig. S5B), further supporting the hypothesis that cluster formation and effector-response observed is associated with protective immunity.

To identify the cell population critical for mediating liver-stage protection, cell depletion was performed after prime-target immunization (Ad.OVA-i.m./Np.OVA-i.v.). Depletion of CD8⁺ T-cells prior to OVA-spz challenge completely ablated vaccine efficacy (**Fig. 2F**; fig. S6). Furthermore, protection was found to scale in a dose-dependent manner, with higher numbers of liver Ag-specific CD8⁺ T-cells correlating with increased protection (fig. S7). CD4⁺ T-cells, although also present in large numbers around infected hepatocytes (**Fig. 2B, C**), were largely dispensable for protection against OVA-spz challenge (**Fig. 2F**). Together, the imaging and depletion studies further confirmed a critical role for CD8⁺ T-cells in mediating protection against liver-stage malaria.

CD8⁺ T-cells expressing markers of tissue residency are found to be sufficient at mediating protection

Recent data suggest that an important contribution to local immunity is provided by T_{RM} (28, 32, 42). We postulated that protection from OVA-spz challenge by prime-target immunization could be the result of such CD8⁺ liver-resident T-cells, rather than the protection due to acute recruitment of memory effector cells from lymphoid sites. To examine whether T_{RM} cells were present in the liver after vaccination with Ad.OVA-i.m./Np.OVA-i.v. or Ad.OVA-i.m., we employed quantitative histo-cytometric analysis (43) of liver tissue 5-weeks after Np.OVA-i.v. administration or only Ad.OVA-i.m. Following prime-target immunization, an increase in T_{RM} (CXCR6⁺ CD69⁺ CD44⁺) (32) and T-effector memory cells (T_{EM}, CXCR6⁻ CD69⁻ CD44⁺) were observed predominantly in the liver sinusoids (as previously noted (20, 44, 45)), compared to Ad.OVA-i.m. vaccinated animals (fig. S8, **Fig. 3A, B**). Not previously described was an increase in small clusters containing T_{EM}, T_{RM}, CD4⁺ T-cells, and F4/80 MHC-II⁺ Kupffer cells in the tissue parenchyma of Ad.OVA-i.m./Np.OVA-i.v. vaccinated animals, appearing similar to Memory Lymphocyte Clusters (42) (**Fig. 3A, C, D**). In addition to T_{RM} marker expression, after isolation and transfer to naïve recipient hosts, liver CD69⁺ CD8⁺ T-cells preferentially homed back to the liver, even when transferring bulk liver lymphocytes (fig. S9). To determine the durability of prime-target vaccine, mice were immunised and lymphocytes were isolated at different time intervals post-vaccination and a population of liver-localized Ag-specific CD8⁺ T-cells, expressing markers of tissue residency, could be detected for at least 36 days following Ad.OVA-i.m./Np.OVA-i.v. immunization (**Fig. 3E, F**; fig. S10A, B). Importantly, protection in vaccinated animals was durable with 100% sterile efficacy at 2-months and 70% at 6-months post-vaccination (**Fig. 3G**; fig. S10C).

Although many of the T-cells found in the liver 5-weeks after Np boost were T_{RM}, to further assess if immunity resulted from T_{EM} drawn into the liver upon OVA-spz infection or if liver T_{RM} were sufficient to mediate protection, mice were challenged under two conditions. Firstly, vaccinated animals were administered FTY720 (fig. S11A) and splenectomised (removing the remaining splenic red pulp T-effector pool) (46) (**Fig. 3H**). Secondly, FTY720 alone or in combination with anti-CD8 depleting antibodies was administered (fig. S11B). In either experimental setting, T_{RM} were found to be sufficient

1 for protection in liver-stage malaria. In summary, these data suggest that prime-target
2 vaccination using Np.OVA-i.v. induces a high frequency of T_{RM} that are stably maintained
3 over time and sufficient for protection against liver-stage malaria.

4 5 **Viral vectors were found to be an effective alternative targeting approach to PLGA** 6 **nanoparticles**

7 Although Nps are being examined in many pre-clinical vaccine studies, non-
8 replicating viral vectors have been utilized i.m. safely in many thousands of humans in a
9 wide variety of vaccines against several infectious diseases generating impressive T cell
10 immunogenicity (47). Hence we examined whether a similar protection to Nps could be
11 obtained using a recombinant viral vector to target. Clinically tested human and
12 chimpanzee adenoviral and MVA vectors, known to induce CD8⁺ T-cell responses in
13 humans (8), were evaluated. Bioluminescent imaging revealed that adeno- as well as
14 MVA-vectors were highly liver-tropic after i.v.-administration (**Fig. 4A, B**), and thus
15 delivered antigen to hepatic tissue. Vaccine regimens involving combinations of Ad.OVA-
16 i.m./Ad.OVA-i.v. or Ad.OVA-i.m./MVA.OVA-i.v. were able to generate substantial
17 numbers of Ag-specific CD8⁺ T-cells in the liver (**Fig. 4C**). A high percentage of these
18 cells also produced IFN γ and expressed markers of tissue residency (fig. S12A, B). Vaccine
19 efficacy was assessed following challenge with OVA-spz. Similar to prime-target
20 vaccination with Np.OVA-i.v., the protective response mediated by prime-target
21 vaccination with viral i.v. boost was found to be dose-dependent, and antigen-specific (fig.
22 S12C, D). As expected, boosting using Ad.OVA-i.m. or MVA.OVA-i.m. vectors failed to
23 induce strong protection. However, boosting with Ad.OVA-i.v. or MVA-OVA-i.v. resulted
24 in 80% sterile protection for 2-months post vaccination (**Fig. 4D**; fig. S12E).

25 26 **Sterile protection with clinically relevant antigens is achieved in mice**

27 To investigate the protective efficacy of a prime and target strategy with clinically
28 relevant antigens, we immunized mice with three *P. falciparum* liver-stage antigens:
29 thrombospondin-related adhesion protein (*PfTRAP*), liver-stage antigen 1 (*PfLSA1*) and
30 liver-stage associated protein 2 (*PfLSAP2*), all now in clinical development. Mice were
31 also immunized with the immunodominant *P. berghei* Pb9 epitope (48) from CSP, present

in the clinically tested ME-TRAP construct (8, 11). Each Ag was individually expressed in the ChAd63 vector administered i.m. Two weeks later, mice were vaccinated with either MVA-i.m. or ChAd63-i.v. expressing the same cognate antigen and animals were challenged 3-weeks later with wild-type (WT) or Tg-spz expressing the *P. falciparum* Ag in *P. berghei* (49, 50). Vaccination using a recombinant ChAd63-i.m./ChAd63-i.v. regimen expressing all these *P. falciparum* Ag singly conferred 100% sterile protection against the antigen-matched Tg-spz (**Fig. 5A-D**). Targeting with Np.Pb9-i.v. or ChAd63.ME-TRAP-i.v. resulted in equivalent levels of protection against WT *P. berghei*. Collectively, these results demonstrated that effective protection through prime-target immunization could be achieved pre-clinically with clinical-stage viral vectors. To further assess the translatable potential of viral vector prime-target immunization against other hepatic diseases, high numbers of liver Ag-specific T-cells specific to hepatitis B surface antigen (HBsAg) could also be successfully induced (fig. S13), indicating the potential flexibility of this platform.

A Phase I clinical trial involving intravenous administration of viral vectors showed a positive safety profile in humans

Following the completion of satisfactory toxicology testing, we performed a small Phase I clinical trial with the primary objective of assessing safety and tolerability of i.v. administration of ChAd63.ME-TRAP in healthy human volunteers. This dose escalation trial involved 3 subjects per dose group (Group 1 5×10^8 , Group 2 5×10^9 , Group 3 5×10^{10} viral particles (vp)), with 90 days of follow up. Following vaccination, adverse events (AEs) local to the injection site were rare, and systemic AEs more frequent, but still mild to moderate (**Fig. 6A**). A single severe AE (fever) was seen in one Group 3 volunteer who incidentally developed clinically evident mumps (confirmed serologically) 4-5 days after immunization, infection almost certainly having occurred prior to immunization. Close monitoring of laboratory parameters was followed to assess for any derangement of liver function and disseminated intravascular coagulation (DIC) (fig. S14A) (51-53). No transaminitis was observed; three volunteers had a mild transient rise in d-dimer within normal limits. Overall, ChAd63.ME-TRAP-i.v. was well tolerated, comparing favorably with previously reported i.m. administrations (54).

Following ChAd63.ME-TRAP-i.v. administration *ex-vivo* IFN γ ELISPOT responses peaked at day 14 post immunization and, appeared to be maintained up until day 42 post vaccination (**Fig. 6B**). Although low vaccine doses resulted in marginal responses Group 3 responders showed higher magnitudes (**Fig. 6C**), at least comparable to a previous clinical trial where the same dose of ChAd63.ME-TRAP was administered i.m. (54). The non-responder in Group 3 was the subject found to be incubating mumps at the time of immunization. Mumps virus has been reported to potentially interfere with innate immunity by inhibiting TNF α -mediated apoptosis and NF- κ B activation (55). The frequency of T_{EM} cells detected in peripheral blood by flow cytometry was increased 14 days after vaccination by either route (fig. S14B) and expression of IFN γ or TNF α on CD8⁺ T-cells did not differ by vaccination route (fig. S14C). These initial safety and immunogenicity data support future clinical testing of the efficacy of prime-target regimens in humans.

Discussion

We describe the use of a prime and target immunization approach that provides a pre-erythrocytic vaccine strategy mediating sterile protection via CD8⁺ T-cells. This vaccination regimen, requiring just a single viral vector, primes endogenous CD8⁺ T-cells in peripheral tissue and subsequently targets them to the liver by expressing suitable amounts of antigen in this organ. This results in a ten-fold increase of Ag-specific CD8⁺ T-cells localized in the liver. Following OVA-spz challenge mice showed 100% sterile protection at 2-months post vaccination and 70% when challenged at 6-months. Hence, prime and target vaccination appears to generate sufficient Ag-specific CD8⁺ T-cells to mediate relatively durable protection in mice, a crucial feature for translatable vaccines.

Using multi-parameter imaging techniques, polyclonal CD8⁺ T-cell were found to cluster in large numbers around infected hepatocytes when cognate antigen was present, producing IFN γ and Granzyme B, as previously observed (39-41). Despite the high frequencies of CD4⁺ T-cells around infected hepatocytes, our depletion studies confirmed a known paradigm in the literature that CD8⁺ T-cells are essential for mediating protection (56). Furthermore, sterile protection was found to scale in a dose-dependent manner with higher levels of Ag-specific CD8⁺ T-cells in the liver correlating with increased protection.

Following careful *in situ* phenotypic characterization, Ag-specific CD8⁺ T-cells were found to express markers of T_{RM} (CXCR6⁺ CD69⁺). In agreement with previous work (25, 32), the number of liver T_{RM} CD8⁺ T-cells correlated with protection in the context of liver-stage malaria, clearly showing that this subset of cells is necessary to mediate protection. Here, we wanted to address whether T_{RM} alone were sufficient in mediating protection to spz challenge or if they acted as sentinels, recruiting other CD8⁺ T-cells from the circulation or dLN. To address this, we used a combination of chemical (FTY720) and surgical (splenectomy) methods to inhibit or remove T-effector CD8⁺ lymphocytes from the circulation. Notably, T_{RM} were found to be sufficient in mediating protection against liver-stage malaria suggesting that a resident Ag-specific T_{RM} population was indeed effective at eliminating infected hepatocytes. Hence, together with previous work (25, 26, 32), liver T_{RM} appear both necessary and sufficient to mediate protection against liver-stage malaria. It should be noted, however, that we cannot completely exclude the contribution of circulating Ag-specific CD8⁺ T-cells during a natural liver-stage infection. In the case of vaccination regimens such as ChAd63-i.m./ChAd63-i.v. that result in high numbers of spleen T_{EM}, circulating Ag-specific CD8⁺ T-cells may also play a role in mediating protection. In fact, vaccine regimens that induce high numbers of T_{RM} and circulating T_{EM} might show improved efficacy in human clinical trials.

In addition to nanoparticles, i.v. administration of leading clinical recombinant viral vectors, ChAd and MVA, both sterilely protected 80% of mice 2-months post vaccination. Moreover, complete sterile protection with the leading *P. falciparum* pre-erythrocytic antigens PfTRAP, PfLSA1, and PfLSAP2 expressed in a ChAd vector was also observed. The efficacy and immunogenicity of these viral-vector and antigen combinations have so far only been assessed in mice and further work will be required to evaluate in humans.

A range of experimental immunization strategies have been reported to induce Ag-specific T_{RM}: Ag-coupled to mAb (27), chemokine administration (28, 29), inflammatory agents (30), or inactivated pathogens (31). However, unlike other vaccine strategies with substantial translational challenges, non-replicating viral vectors are excellent targeting agents, with clinically tested ChAd offering a particularly suitable clinical option with only one recombinant adenoviral vector required and no change in immunogen necessary, significantly simplifying biomanufacturing. Furthermore, because viral vectors are able to

express any antigen of interest, prime-target vaccination is an adaptable vaccine platform potentially protective against other hepatic diseases.

To assess the likely safety and feasibility of incorporating i.v. viral-vectored vaccination in a deployable prime-target regimen, a small phase I study was conducted, testing safety and immunogenicity of a single ChAd63.ME-TRAP-i.v. dose. The i.v. administration of ChAd63.ME-TRAP was well tolerated with no severe vaccine-related AEs. Close monitoring of laboratory parameters revealed no indication of hepatocellular damage or significant inflammation previously seen with i.v. administration of some adenoviruses in the setting of oncolytic virotherapy using much higher doses (51-53). This represents a significant step in bringing prime-target vaccination into clinical testing. Furthermore, the immunogenicity of a single i.v. vaccine dose as assayed by a standard *ex vivo* ELISPOTs appear encouraging. Taken together, this prime-target approach substantially improves current antigen-based liver-stage malaria vaccines, providing a potent and flexible immunization platform.

Materials and Methods

Study Design

The study aimed at developing a translatable malaria vaccine strategy that resulted in sterile protection in a *P. berghei* spz challenge model of the disease. Accordingly, experiments were performed to test various vaccination models. The *n* for these experiments was determined by performing an *a priori* power analysis, used in estimating sufficient sample sizes to achieve adequate power. No randomization or blinding was performed; mice were excluded from experiments only when tail i.v. injections were not performed successfully, determined at the time of vaccination. The endpoint of 1% parasitemia was chosen as a humane standard widely used in the literature. Following 1% parasitemia, malaria symptoms in mice only progress in severity, inevitability leading to death, with animals unable to recover from the infection. Following challenge experiments, flow cytometry and IHC experiments were performed to investigate the mechanism of protection. The hypothesis that vaccine regimens would affect sterile protection was specified in advance, and a Linear Mixed Model (LMM) was performed, which takes into account variation between mice, experiments, or tissue slices (in the context of IHC) as

potential sources of random variation in addition to fixed effects (explanatory variables). Most experiments were performed with the model antigen OVA; however, clinical malaria vaccine candidates were also tested with this platform and found to mediate sterile protection. Primary data are located in table S1. A small Phase I clinical trial was performed to assess the safety and tolerability of i.v. administration of ChAd63 ME-TRAP in healthy malaria-naïve volunteers.

Experimental animals and immunizations

All animal work was approved by the University of Oxford Animal Care and Ethical Review Committee and performed under License 30/2889 or P9804B4F1 or approved by the NIAID Animal Care and Use Committee (NIH) under the LSB-4E animal license. Male and female C57BL/6 CD45.2 and CD45.1, BALB/c, and ICR (CD-1) mice (Envigo) and B6.SJL-Cd45a(Ly5a)/Nai, C57BL/6Nai-[Tg]TCR OTI-[KO]RAG1 (57) (Taconic) and Ubiquitin-tdTomato transgenic mice (generous gift from R. Tsien, University of California SD), of at least six weeks of age were used. Mice were immunized i.m. into the musculus tibialis with a dose of 1×10^8 infectious units (iu) for Ad, or 1×10^6 plaque forming units (pfu) for MVA. For tail vein i.v. administration, the following doses were administered: 1×10^9 iu for Ad vectors or 1×10^6 pfu for MVA vectors. Description of viral vectors used found in Supplementary Materials.

Intracellular cytokine staining assay

ICS was performed as previously described (58), see Supplementary Materials for greater detail. In short, liver lymphocytes were isolated following *in situ* PBS perfusion of the liver via the portal vein and stimulated for six hours at 37°C with appropriate peptides at a final concentration of 1 µg/ml (OVA peptides used were SIINFEKL and/or ISQAVHAAHAEINEAGR, HBsAg H2-L^d dominant peptide IPQSLDSWWTSL, ProImmune), 1 µg/ml GolgiPlug (BD) or left unstimulated in complete media and GolgiPlug for control.

Cells were subsequently surface stained on ice (see Supplementary Materials for detailed antibody list) and samples were acquired using an LSR II flow cytometer (BD) or

LSR Fortessa (BD) and analysed using FlowJo v9 or v10.1 (Tree Star Inc.). Lymphocytes were gated on live, size, singlet and subsequently gated on CD8⁺ T-cells.

Methodological information regarding clinical trial (NCT03084289):

See Data File for details on clinical trial VAC064 (NCT03084289).

Statistical Analysis

Prism v5.0c (Graphpad, USA) and R-studio were used for all analyses. Median values with interquartile range are shown in all graphs, unless otherwise stated. Data were tested for normality and homoskedasticity and where appropriate, data was log-transformed. For these reasons, all experiments showing total cell counts were log-transformed. When data were pooled from multiple experiments, a multi-level analysis using a linear mixed model (LMM) from R. Hence, *lme4* was used to perform a linear mixed effects analysis of the relationship between measured vaccine output (dependent variable) and vaccination (independent variable) (59). Fixed effects used were determined to be the variable/treatment under examination, while random effects were variability caused by experiment-to-experiment variation (specified for each figure in figure legend). A p-value was then obtained by performing a likelihood of ratio-test. This was done by comparing the full model and to a null model (lacking the fixed-variable of interest) with an ANOVA. If significance was found from running a LMM, One-Way or Two-way ANOVA was performed, with multiple-comparisons corrected using a Bonferroni's *post hoc* test. Survival in challenge experiments is presented using Kaplan-Meier curves and significance tested using the Log-Rank (Mantel-Cox) Test.

List of Supplementary Materials

Supplementary Materials and Methods

Supplementary Data fig. S1-14

S1: Intravenous Np localization and OTI activation in liver tissue following i.v. and i.m. Np.APC-OVA administration.

S2: Schematic representation of prime and target vaccination regimen, efficacy and immunogenicity.

S3: Total Pen⁺ CD8⁺ T-cell numbers in the liver are not affected by FTY720 administration.

S4: T-cell recruitment to the liver is dependent in part on the dose of PLGA-OVA administered.

S5: A prime and target approach protects mice against challenge with transgenic *P. berghei* OVA-spz only when mice are vaccinated against OVA.

S6: Flow plots showing representative lymphocyte depletion following administration of depleting antibodies.

S7: Higher numbers of liver Pen⁺ CD8⁺ T-cells correlated with greater protection following spz challenge.

S8: Histo-cytometric analysis of liver sections.

S9: Liver Pen⁺ CD8⁺ T-cells preferentially home back to liver in recipient mice.

S10: Gating strategy showing T_{RM} phenotype of Pen⁺ T-cells in liver following Np administration and long-term efficacy data.

S11: Flow plots showing representative lymphocyte depletion and inhibition of egress from lymphatics following administration of depleting antibody and FTY720.

S12: A prime and target approach using viral vectors as targeting agents shows similar immunization profile to PLGA Np targeting.

S13: A prime and target approach using viral vectors generates high numbers of HBsAg CD8⁺ T-cells in the liver.

S14: Safety and human immune responses to immunization with ChAd63 ME-TRAP i.v.

Movie S1: Video showing the internalization of Np.APC-OVA within Kupffer cells.

Table S1: Primary data file

References and Notes

1. S. Bhatt, D. J. Weiss, E. Cameron, D. Bisanzio, B. Mappin, U. Dalrymple, K. E. Battle, C. L. Moyes, A. Henry, P. A. Eckhoff, E. A. Wenger, O. Briet, M. A. Penny, T. A. Smith, A. Bennett, J. Yukich, T. P. Eisele, J. T. Griffin, C. A. Fergus, M. Lynch, F. Lindgren, J. M. Cohen, C. L. Murray, D. L. Smith, S. I. Hay, R. E. Cibulskis, P. W. Gething, The effect of malaria control on *Plasmodium falciparum* in Africa between 2000 and 2015. *Nature* **526**, 207-211 (2015).

2. Global. WHO declares emergency against AIDS, TB, malaria. *AIDS Policy Law* **21**, 5 (2006).
3. S. T. Agnandji, B. Lell, S. S. Soulanoudjingar, J. F. Fernandes, et al., First results of phase 3 trial of RTS,S/AS01 malaria vaccine in African children. *N Engl J Med* **365**, 1863-1875 (2011).
4. L. Schofield, J. Villaquiran, A. Ferreira, H. Schellekens, R. Nussenzweig, V. Nussenzweig, Gamma interferon, CD8+ T cells and antibodies required for immunity to malaria sporozoites. *Nature* **330**, 664-666 (1987).
5. W. R. Weiss, M. Sedegah, R. L. Beaudoin, L. H. Miller, M. F. Good, CD8+ T cells (cytotoxic/suppressors) are required for protection in mice immunized with malaria sporozoites. *Proc Natl Acad Sci U S A* **85**, 573-576 (1988).
6. A. V. Hill, A. Reyes-Sandoval, G. O'Hara, K. Ewer, A. Lawrie, A. Goodman, A. Nicosia, A. Folgori, S. Colloca, R. Cortese, S. C. Gilbert, S. J. Draper, Prime-boost vectored malaria vaccines: progress and prospects. *Hum Vaccin* **6**, 78-83 (2010).
7. S. Li, M. Rodrigues, D. Rodriguez, J. R. Rodriguez, M. Esteban, P. Palese, R. S. Nussenzweig, F. Zavala, Priming with recombinant influenza virus followed by administration of recombinant vaccinia virus induces CD8+ T-cell-mediated protective immunity against malaria. *Proc Natl Acad Sci U S A* **90**, 5214-5218 (1993).
8. K. J. Ewer, G. A. O'Hara, C. J. Duncan, K. A. Collins, S. H. Sheehy, A. Reyes-Sandoval, A. L. Goodman, N. J. Edwards, S. C. Elias, F. D. Halstead, R. J. Longley, R. Rowland, I. D. Poulton, S. J. Draper, A. M. Blagborough, E. Berrie, S. Moyle, N. Williams, L. Siani, A. Folgori, S. Colloca, R. E. Sinden, A. M. Lawrie, R. Cortese, S. C. Gilbert, A. Nicosia, A. V. Hill, Protective CD8+ T-cell immunity to human malaria induced by chimpanzee adenovirus-MVA immunisation. *Nat Commun* **4**, 2836 (2013).
9. A. Reyes-Sandoval, T. Berthoud, N. Alder, L. Siani, S. C. Gilbert, A. Nicosia, S. Colloca, R. Cortese, A. V. Hill, Prime-boost immunization with adenoviral and modified vaccinia virus Ankara vectors enhances the durability and polyfunctionality of protective malaria CD8+ T-cell responses. *Infect Immun* **78**, 145-153 (2010).
10. S. Capone, A. Reyes-Sandoval, M. Naddeo, L. Siani, V. Ammendola, C. S. Rollier, A. Nicosia, S. Colloca, R. Cortese, A. Folgori, A. V. Hill, Immune responses against a liver-stage malaria antigen induced by simian adenoviral vector AdCh63 and MVA prime-boost immunisation in non-human primates. *Vaccine* **29**, 256-265 (2010).
11. C. Ogowang, D. Kimani, N. J. Edwards, R. Roberts, J. Mwacharo, G. Bowyer, C. Bliss, S. H. Hodgson, P. Njuguna, N. K. Viebig, A. Nicosia, E. Gitau, S. Douglas, J. Illingworth, K. Marsh, A. Lawrie, E. B. Imoukhuede, K. Ewer, B. C. Urban, S. H. AV, P. Bejon, M. Group, Prime-boost vaccination with chimpanzee adenovirus and modified vaccinia Ankara encoding TRAP provides partial protection against *Plasmodium falciparum* infection in Kenyan adults. *Sci Transl Med* **7**, 286re285 (2015).
12. S. Dunachie, A. V. Hill, H. A. Fletcher, Profiling the host response to malaria vaccination and malaria challenge. *Vaccine* **33**, 5316-5320 (2015).
13. I. A. Cockburn, Y. C. Chen, M. G. Overstreet, J. R. Lees, N. van Rooijen, D. L. Farber, F. Zavala, Prolonged antigen presentation is required for optimal CD8+ T

cell responses against malaria liver stage parasites. *PLoS Pathog* **6**, e1000877 (2010).

14. I. N. Crispe, M. Giannandrea, I. Klein, B. John, B. Sampson, S. Wuensch, Cellular and molecular mechanisms of liver tolerance. *Immunol Rev* **213**, 101-118 (2006).
15. U. Protzer, M. K. Maini, P. A. Knolle, Living in the liver: hepatic infections. *Nat Rev Immunol* **12**, 201-213 (2012).
16. S. S. Tay, Y. C. Wong, D. M. McDonald, N. A. Wood, B. Roediger, F. Sierro, C. McGuffog, I. E. Alexander, G. A. Bishop, J. R. Gamble, W. Weninger, G. W. McCaughan, P. Bertolino, D. G. Bowen, Antigen expression level threshold tunes the fate of CD8 T cells during primary hepatic immune responses. *Proc Natl Acad Sci USA* **111**, E2540-2549 (2014).
17. T. Gebhardt, L. M. Wakim, L. Eidsmo, P. C. Reading, W. R. Heath, F. R. Carbone, Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus. *Nat Immunol* **10**, 524-530 (2009).
18. D. Masopust, D. Choo, V. Vezys, E. J. Wherry, J. Duraiswamy, R. Akondy, J. Wang, K. A. Casey, D. L. Barber, K. S. Kawamura, K. A. Fraser, R. J. Webby, V. Brinkmann, E. C. Butcher, K. A. Newell, R. Ahmed, Dynamic T cell migration program provides resident memory within intestinal epithelium. *J Exp Med* **207**, 553-564 (2010).
19. D. Masopust, V. Vezys, E. J. Wherry, D. L. Barber, R. Ahmed, Cutting edge: gut microenvironment promotes differentiation of a unique memory CD8 T cell population. *J Immunol* **176**, 2079-2083 (2006).
20. E. M. Steinert, J. M. Schenkel, K. A. Fraser, L. K. Beura, L. S. Manlove, B. Z. Igyarto, P. J. Southern, D. Masopust, Quantifying Memory CD8 T Cells Reveals Regionalization of Immunosurveillance. *Cell* **161**, 737-749 (2015).
21. L. M. Wakim, N. Gupta, J. D. Mintern, J. A. Villadangos, Enhanced survival of lung tissue-resident memory CD8(+) T cells during infection with influenza virus due to selective expression of IFITM3. *Nat Immunol* **14**, 238-245 (2013).
22. T. Gebhardt, P. G. Whitney, A. Zaid, L. K. Mackay, A. G. Brooks, W. R. Heath, F. R. Carbone, S. N. Mueller, Different patterns of peripheral migration by memory CD4+ and CD8+ T cells. *Nature* **477**, 216-219 (2011).
23. J. M. Schenkel, K. A. Fraser, L. K. Beura, K. E. Pauken, V. Vezys, D. Masopust, T cell memory. Resident memory CD8 T cells trigger protective innate and adaptive immune responses. *Science* **346**, 98-101 (2014).
24. S. W. Tse, I. A. Cockburn, H. Zhang, A. L. Scott, F. Zavala, Unique transcriptional profile of liver-resident memory CD8+ T cells induced by immunization with malaria sporozoites. *Genes Immun* **14**, 302-309 (2013).
25. S. W. Tse, A. J. Radtke, D. A. Espinosa, I. A. Cockburn, F. Zavala, The chemokine receptor CXCR6 is required for the maintenance of liver memory CD8(+) T cells specific for infectious pathogens. *J Infect Dis* **210**, 1508-1516 (2014).
26. J. E. Epstein, K. Tewari, K. E. Lyke, B. K. Sim, P. F. Billingsley, M. B. Laurens, A. Gunasekera, S. Chakravarty, E. R. James, M. Sedegah, A. Richman, S. Velmurugan, S. Reyes, M. Li, K. Tucker, A. Ahumada, A. J. Ruben, T. Li, R. Stafford, A. G. Eappen, C. Tamminga, J. W. Bennett, C. F. Ockenhouse, J. R. Murphy, J. Komisar, N. Thomas, M. Loyevsky, A. Birkett, C. V. Plowe, C. Loucq, R. Edelman, T. L. Richie, R. A. Seder,

- 1 S. L. Hoffman, Live attenuated malaria vaccine designed to protect through
2 hepatic CD8(+) T cell immunity. *Science* **334**, 475-480 (2011).
- 3 27. L. M. Wakim, J. Smith, I. Caminschi, M. H. Lahoud, J. A. Villadangos, Antibody-
4 targeted vaccination to lung dendritic cells generates tissue-resident memory
5 CD8 T cells that are highly protective against influenza virus infection. *Mucosal*
6 *Immunol* **8**, 1060-1071 (2015).
- 7 28. H. Shin, A. Iwasaki, A vaccine strategy that protects against genital herpes by
8 establishing local memory T cells. *Nature* **491**, 463-467 (2012).
- 9 29. J. E. Kohlmeier, S. C. Miller, J. Smith, B. Lu, C. Gerard, T. Cookenham, A. D.
10 Roberts, D. L. Woodland, The chemokine receptor CCR5 plays a key role in the
11 early memory CD8+ T cell response to respiratory virus infections. *Immunity* **29**,
12 101-113 (2008).
- 13 30. L. K. Mackay, A. T. Stock, J. Z. Ma, C. M. Jones, S. J. Kent, S. N. Mueller, W. R.
14 Heath, F. R. Carbone, T. Gebhardt, Long-lived epithelial immunity by tissue-
15 resident memory T (TRM) cells in the absence of persisting local antigen
16 presentation. *Proc Natl Acad Sci U S A* **109**, 7037-7042 (2012).
- 17 31. G. Stary, A. Olive, A. F. Radovic-Moreno, D. Gondek, D. Alvarez, P. A. Basto, M.
18 Perro, V. D. Vrbanc, A. M. Tager, J. Shi, J. A. Yethon, O. C. Farokhzad, R. Langer, M.
19 N. Starnbach, U. H. von Andrian, VACCINES. A mucosal vaccine against Chlamydia
20 trachomatis generates two waves of protective memory T cells. *Science* **348**,
21 aaa8205 (2015).
- 22 32. D. Fernandez-Ruiz, W. Y. Ng, L. E. Holz, J. Z. Ma, A. Zaid, Y. C. Wong, L. S. Lau, V.
23 Mollard, A. Cozijnsen, N. Collins, J. Li, G. M. Davey, Y. Kato, S. Devi, R. Skandari, M.
24 Pauley, J. H. Manton, D. I. Godfrey, A. Braun, S. S. Tay, P. S. Tan, D. G. Bowen, F.
25 Koch-Nolte, B. Rissiek, F. R. Carbone, B. S. Crabb, M. Lahoud, I. A. Cockburn, S. N.
26 Mueller, P. Bertolino, G. I. McFadden, I. Caminschi, W. R. Heath, Liver-Resident
27 Memory CD8+ T Cells Form a Front-Line Defense against Malaria Liver-Stage
28 Infection. *Immunity* **45**, 889-902 (2016).
- 29 33. A. K. Mohammad, J. J. Reineke, Quantitative detection of PLGA nanoparticle
30 degradation in tissues following intravenous administration. *Mol Pharm* **10**,
31 2183-2189 (2013).
- 32 34. S. Stoll, J. Delon, T. M. Brotz, R. N. Germain, Dynamic imaging of T cell-
33 dendritic cell interactions in lymph nodes. *Science* **296**, 1873-1876 (2002).
- 34 35. J. W. Lin, T. N. Shaw, T. Annoura, A. Fougere, P. Bouchier, S. Chevalley-Maurel,
35 H. Kroeze, B. Franke-Fayard, C. J. Janse, K. N. Couper, S. M. Khan, The subcellular
36 location of ovalbumin in Plasmodium berghei blood stages influences the
37 magnitude of T-cell responses. *Infect Immun* **82**, 4654-4665 (2014).
- 38 36. S. Mandala, R. Hajdu, J. Bergstrom, E. Quackenbush, J. Xie, J. Milligan, R.
39 Thornton, G. J. Shei, D. Card, C. Keohane, M. Rosenbach, J. Hale, C. L. Lynch, K.
40 Rupprecht, W. Parsons, H. Rosen, Alteration of lymphocyte trafficking by
41 sphingosine-1-phosphate receptor agonists. *Science* **296**, 346-349 (2002).
- 42 37. S. P. Kasturi, I. Skountzou, R. A. Albrecht, D. Koutsonanos, T. Hua, H. I. Nakaya,
43 R. Ravindran, S. Stewart, M. Alam, M. Kwissa, F. Villinger, N. Murthy, J. Steel, J.
44 Jacob, R. J. Hogan, A. Garcia-Sastre, R. Compans, B. Pulendran, Programming the
45 magnitude and persistence of antibody responses with innate immunity. *Nature*
46 **470**, 543-547 (2011).

38. S. Bruno, Z. Darzynkiewicz, Cell cycle dependent expression and stability of the nuclear protein detected by Ki-67 antibody in HL-60 cells. *Cell Prolif* **25**, 31-40 (1992).
39. I. A. Cockburn, R. Amino, R. K. Kelemen, S. C. Kuo, S. W. Tse, A. Radtke, L. MacDaniel, V. V. Ganusov, F. Zavala, R. Menard, In vivo imaging of CD8+ T cell-mediated elimination of malaria liver stages. *Proc Natl Acad Sci U S A* **110**, 9090-9095 (2013).
40. K. Kimura, D. Kimura, Y. Matsushima, M. Miyakoda, K. Honma, M. Yuda, K. Yui, CD8+ T cells specific for a malaria cytoplasmic antigen form clusters around infected hepatocytes and are protective at the liver stage of infection. *Infect Immun* **81**, 3825-3834 (2013).
41. S. L. Hoffman, D. Isenbarger, G. W. Long, M. Sedegah, A. Szarfman, L. Waters, M. R. Hollingdale, P. H. van der Meide, D. S. Finbloom, W. R. Ballou, Sporozoite vaccine induces genetically restricted T cell elimination of malaria from hepatocytes. *Science* **244**, 1078-1081 (1989).
42. N. Iijima, A. Iwasaki, T cell memory. A local macrophage chemokine network sustains protective tissue-resident memory CD4 T cells. *Science* **346**, 93-98 (2014).
43. M. Y. Gerner, W. Kastenmuller, I. Ifrim, J. Kabat, R. N. Germain, Histocytometry: a method for highly multiplex quantitative tissue imaging analysis applied to dendritic cell subset microanatomy in lymph nodes. *Immunity* **37**, 364-376 (2012).
44. H. A. McNamara, Y. Cai, M. V. Wagle, Y. Sontani, C. M. Roots, L. A. Miosge, J. H. O'Connor, H. J. Sutton, V. V. Ganusov, W. R. Heath, P. Bertolino, C. G. Goodnow, I. A. Parish, A. Enders, I. A. Cockburn, Up-regulation of LFA-1 allows liver-resident memory T cells to patrol and remain in the hepatic sinusoids. *Sci Immunol* **2**, (2017).
45. M. Cabrera, L. L. Pewe, J. T. Harty, U. Frevort, In vivo CD8+ T cell dynamics in the liver of Plasmodium yoelii immunized and infected mice. *PLoS One* **8**, e70842 (2013).
46. T. I. Arnon, J. G. Cyster, Blood, sphingosine-1-phosphate and lymphocyte migration dynamics in the spleen. *Curr Top Microbiol Immunol* **378**, 107-128 (2014).
47. K. J. Ewer, T. Lambe, C. S. Rollier, A. J. Spencer, A. V. Hill, L. Dorrell, Viral vectors as vaccine platforms: from immunogenicity to impact. *Curr Opin Immunol* **41**, 47-54 (2016).
48. P. Romero, J. L. Maryanski, G. Corradin, R. S. Nussenzweig, V. Nussenzweig, F. Zavala, Cloned cytotoxic T cells recognize an epitope in the circumsporozoite protein and protect against malaria. *Nature* **341**, 323-326 (1989).
49. R. J. Longley, A. M. Salman, M. G. Cottingham, K. Ewer, C. J. Janse, S. M. Khan, A. J. Spencer, A. V. Hill, Comparative assessment of vaccine vectors encoding ten malaria antigens identifies two protective liver-stage candidates. *Sci Rep* **5**, 11820 (2015).
50. A. M. Salman, C. M. Mogollon, J. W. Lin, F. J. van Pul, C. J. Janse, S. M. Khan, Generation of Transgenic Rodent Malaria Parasites Expressing Human Malaria Parasite Proteins. *Methods Mol Biol* **1325**, 257-286 (2015).

51. J. Nemunaitis, C. Cunningham, A. Buchanan, A. Blackburn, G. Edelman, P. Maples, G. Netto, A. Tong, B. Randlev, S. Olson, D. Kirn, Intravenous infusion of a replication-selective adenovirus (ONYX-015) in cancer patients: safety, feasibility and biological activity. *Gene Ther* **8**, 746-759 (2001).
52. T. Reid, E. Galanis, J. Abbruzzese, D. Sze, L. M. Wein, J. Andrews, B. Randlev, C. Heise, M. Uprichard, M. Hatfield, L. Rome, J. Rubin, D. Kirn, Hepatic arterial infusion of a replication-selective oncolytic adenovirus (dl1520): phase II viral, immunologic, and clinical endpoints. *Cancer Res* **62**, 6070-6079 (2002).
53. E. J. Small, M. A. Carducci, J. M. Burke, R. Rodriguez, L. Fong, L. van Ummersen, D. C. Yu, J. Aimi, D. Ando, P. Working, D. Kirn, G. Wilding, A phase I trial of intravenous CG7870, a replication-selective, prostate-specific antigen-targeted oncolytic adenovirus, for the treatment of hormone-refractory, metastatic prostate cancer. *Mol Ther* **14**, 107-117 (2006).
54. S. H. Hodgson, K. J. Ewer, C. M. Bliss, N. J. Edwards, T. Rampling, N. A. Anagnostou, E. de Barra, T. Havelock, G. Bowyer, I. D. Poulton, S. de Cassan, R. Longley, J. J. Illingworth, A. D. Douglas, P. B. Mange, K. A. Collins, R. Roberts, S. Gerry, E. Berrie, S. Moyle, S. Colloca, R. Cortese, R. E. Sinden, S. C. Gilbert, P. Bejon, A. M. Lawrie, A. Nicosia, S. N. Faust, A. V. Hill, Evaluation of the efficacy of ChAd63-MVA vectored vaccines expressing circumsporozoite protein and ME-TRAP against controlled human malaria infection in malaria-naïve individuals. *J Infect Dis* **211**, 1076-1086 (2015).
55. S. Franz, P. Rennert, M. Woznik, J. Grutzke, A. Ludde, E. M. Arriero Pais, T. Finsterbusch, H. Geyer, A. Mankertz, N. Friedrich, Mumps Virus SH Protein Inhibits NF-kappaB Activation by Interacting with Tumor Necrosis Factor Receptor 1, Interleukin-1 Receptor 1, and Toll-Like Receptor 3 Complexes. *J Virol* **91**, (2017).
56. N. W. Schmidt, N. S. Butler, V. P. Badovinac, J. T. Harty, Extreme CD8 T cell requirements for anti-malarial liver-stage immunity following immunization with radiation attenuated sporozoites. *PLoS Pathog* **6**, e1000998 (2010).
57. K. A. Hogquist, S. C. Jameson, W. R. Heath, J. L. Howard, M. J. Bevan, F. R. Carbone, T cell receptor antagonist peptides induce positive selection. *Cell* **76**, 17-27 (1994).
58. A. J. Spencer, M. G. Cottingham, J. A. Jenks, R. J. Longley, S. Capone, S. Colloca, A. Folgori, R. Cortese, A. Nicosia, M. Bregu, A. V. Hill, Enhanced vaccine-induced CD8+ T cell responses to malaria antigen ME-TRAP by fusion to MHC class ii invariant chain. *PLoS One* **9**, e100538 (2014).
59. M. M. Douglas Bates, Ben Bolker, Steve Walker, Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software* **10**, (2015).

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Author Contributions

AG designed, conducted, analyzed experiments, and drafted the manuscript; AAW developed PLGA-Np, designed and interpreted experiments. BRH and AMS conducted challenge experiments and *in vivo* luminescence imaging. AMS, SMK and CJJ provided Tg-spz. SU performed splenectomies. DS designed, conducted and drafted the clinical study with assistance from SS, NV, EB and BA; IP and MB were lead clinical nurses; RR and AL managed ethical and regulatory affairs in relation to NCT03084289. KE designed and analyzed clinical data. JP, GB and DB performed human immunology assays. RNG helped with imaging, data interpretation, and writing of the manuscript. AJS and AVSH designed experiments and interpreted data. All authors contributed and approved the manuscript.

Competing interests: AVSH, AG, AAW and AS are inventors on a patent application PCT/GB2017/051009 submitted by the Oxford University Innovation Limited that covers prime and target vaccination with viral vectors.

Data and Materials Availability: All data associated with the study are present in the paper or Supplementary Materials.

Figure Legends

Fig. 1: Prime and target immunization protects mice against challenge with Tg OVA-spz

C57BL/6 mice (n=6/group) were primed with Ad.OVA-i.m. followed by Np.OVA-i.v. (Ad.OVA-i.m./Np.OVA-i.v.), Np.OVA-i.m. (Ad.OVA-i.m./Np.OVA-i.m.), or no Np.OVA (Ad.OVA-i.m.). Mice were challenged with (A) OVA::Hep17 *P. berghei* spz or (B) OVA::mCherry_{Pbhs70} *P. berghei* spz. Data shown are representative of 2-3 independent experiments; Log-rank Mantel-Cox Test. C57BL/6 mice were primed with either Ad.OVA-i.m. or Np.OVA-i.v. (Np.OVA-i.v.). Ad.OVA-i.m. primed animals were subsequently vaccinated with either OVA protein (Ad.OVA-i.m./OVA-i.v.), Np.OVA-i.m. (Ad.OVA-i.m./Np.OVA-i.m.) or Np.OVA-i.v. (Ad.OVA-i.m./Np.OVA-i.v.), or were left untreated (Ad.OVA-i.m.). Three weeks post Np or Ad.OVA-i.m. vaccination, animals were examined by flow cytometry for (C-E) total Pen⁺ CD8⁺ T-cells in liver, dLN, and spleen; frequency of (F) Pen⁺ and (G) IFN γ ⁺ CD8⁺ T-cells of total CD8⁺ T-cells in liver; (H) total cell count of IFN γ ⁺ CD4⁺ T-cells in liver. Data shown are pooled from 2-4 independent experiments, (n= 7-12/group). Median shown: data were analyzed using a LMM with experiment and mouse as random effects and vaccination and boost as fixed effects. p<0.0001(****); p<0.001(***) ; p<0.01(**); p<0.05(*); not significant (ns).

Fig. 2: Localization and quantification of immune response to spz challenge

Immunofluorescence (IF) images depicting spz in liver 12 hours after challenge (ch:) with an i.v. injection of 5x10⁴ OVA::mCherry_{Pbhs70} *P. berghei* spz (OVA-spz) or mcherry_{Pbhs70} (spz). Mice had been previously vaccinated (v:) with Ad.OVA-i.m./Np.OVA-i.v. or Ad.OVA-i.m. only, and challenged three weeks post Np.OVA-i.v. injection. (A) IF images of liver sections (Maximum Intensity Projection of a 20 μ m z-stack) stained for CSP protein, CD8, CD4, and F4/80; arrowheads denote spz. (B) IF image depicting liver section from Ad.OVA-i.m./Np.OVA-i.v. challenged with OVA-spz. Section stained for CD8, CD4, Ki67, GrzB, IFN γ , and Jojo (nucleus). Right, single color panels. Quantification of IF images: (C) Mean cell count per infectious site (IS) of CD8⁺ and CD4⁺ T-cells (equal numbers of IS imaged per condition). *In situ* frequency of (D) CD8⁺ (E) CD4⁺ T-cell expressing Ki67, GrzB or IFN γ from mice vaccinated with Ad.OVA-i.m./Np.OVA-i.v. and challenged with OVA-spz. IF images are representative of two independent experiments, for a total of n=4 animals per condition (2 liver sections imaged/animal). Median shown from all pooled data: data analyzed using a LMM with experiment and liver section as random effects and vaccination and spz as fixed effects. (F) C57BL/6 mice (n=5/group) were vaccinated with Ad.OVA-i.m./Np.OVA-i.v. Three weeks post Np.OVA-i.v. vaccination mice were administered anti-CD4, anti-CD8 depleting antibodies (Ab) or a combination of both antibodies. Alternatively, IgG2b

isotype control antibody was administered (Iso Ctrl). Mice were subsequently challenged with OVA::Hep17 *P. berghei* spz. Data shown are representative of two independent experiments; Log-rank Mantel-Cox Test. $p < 0.0001$ (****); $p < 0.01$ (**); not significant (ns).

Fig. 3: Prime and target vaccination induces liver T_{RM} stably maintained in the tissue and sufficient in mediating protect

(A) Representative IF images depicting liver tissue of mice vaccinated with Ad.OVA-i.m./Np.OVA-i.v. 5-weeks post Np.OVA-i.v. vaccination. Panel i: CD8, CXCR6, CD4, CD69, CD44, CD3, CD31, and JoJo (nucleus). Panel ii: F4/80, MHC-II, CD8, CD4 and JoJo. Bottom, single color panels. Arrowhead denotes examples of T_{RM} ($CD8^+ CXCR6^+ CD69^+ CD44^+$ T-cell), Asterisk-Arrowhead denotes T_{EM} ($CD8^+ CXCR6^- CD69^- CD44^+$ T-cell) (MIP of a $20\mu m$ z-stack). Quantification of IF images: (B) Total cell count/ $100\mu m^3$ of T_{RM} or T_{EM} in Ad.OVA-i.m. or Ad.OVA-i.m./Np.OVA-i.v. vaccinated animals. Data analyzed using LMM with experiment and tissue slice as random effects, and cell type and vaccination as fixed effects. (C) Total number of clusters/ $100\mu m^3$ in Ad.OVA-i.m. or Ad.OVA-i.m./Np.OVA-i.v. vaccinated animals. Data analyzed using LMM with experiment and cluster as random effects, and vaccination as fixed effect. (D) Frequency of T_{EM} , T_{RM} and $CD4^+$ T-cells of total $CD3^+$ cells in clusters from mice vaccinated with Ad.OVA-i.m./Np.OVA-i.v. Data analyzed using LMM with experiment and cluster as random effects, and vaccination as fixed effect. (A-D) Quantification of IF images is from two independent experiments, for a total of $n=4-6$ animals/condition (2 liver sections imaged/animal), Median shown. (E) C57BL/6 mice were vaccinated Ad.OVA-im/Np.OVA-i.v. or Ad.OVA-i.m. only. Total number of $TCR\alpha^+ (V\alpha 2/V\beta 5)$ liver $CD8^+$ T-cells over time as determined by flow cytometry. Median shown from two pooled experiments: Data analyzed using LMM with experiment as random effect and vaccination and time as fixed effects. At day 36 post Np.OVA-i.v. administration (F) total Pen^+ T-cells determined in liver and spleen. Data shown are pooled from two independent experiments, each symbol representative of an individual mouse ($n=8-9$ /group). Data analyzed using LMM with experiment and mouse as random effects and vaccination as fixed effect. (G) C57BL/6 mice ($n=6$ /group) were vaccinated with Ad.OVA-i.m./Np.OVA-i.v. or Ad.OVA-i.m. Mice were challenged with OVA::Hep17 *P. berghei* spz 2-months, or 6-months post Np.OVA-i.v. vaccination. (H) C57BL/6 mice ($n=6$ /group) were vaccinated with Ad.OVA-i.m./Np.OVA-i.v. Three weeks after Np.OVA administration, mice were either splenectomized or sham operated, and FTY720 was administered. Alternatively, mice were treated with FTY720 and/or anti-CD8 depleting Ab. Mice were subsequently challenged with OVA::Hep17 *P. berghei* spz. Data shown are representative of two independent experiments; Log-rank Mantel-Cox Test. $p < 0.0001$ (****); $p < 0.001$ (***); $p < 0.01$ (**); not significant (ns).

Fig. 4: Viral vectors as targeting agents induce strong protective immunity against challenge with Tg OVA-spz

BALB/c mice ($n=5$ /group) were vaccinated i.v. with Ad, ChAd63 or MVA expressing luciferase (Luc) and imaged at different days (D) post vaccination. (A) Bioluminescence image indicating the location of Luciferase. In red, Region of Interest (ROI) is shown,

with (B) ROI Flux analysis over time. (C) C57BL/6 mice were all primed with Ad.OVA-i.m. and followed two weeks later by MVA.OVA i.m. (Ad.OVA-i.m./MVA.OVA-i.m.), Ad.OVA i.v. (Ad.OVA-i.m./Ad.OVA-i.v.), MVA-OVA-i.v. (Ad.OVA-i.m./MVA.OVA-i.v.), Np.OVA-i.v. (Ad.OVA-i.m./Np.OVA-i.v.) or untreated (Ad.OVA-i.m.). Total Pen⁺ T-cells in liver or spleen. Data shown are pooled from two independent experiments, each symbol representative of individual mouse (n=8-12/group). Median shown; data analyzed with LMM model with experiment and mouse as random variables, and vaccination and boost as fixed variables. All statistical comparisons were made with only statistically significant comparisons shown. (D) C57BL/6 mice (n=6/group) were vaccinated with Ad.OVA-i.m./Ad.OVA-i.m., Ad.OVA-i.m./MVA.OVA-i.m., Ad.OVA-i.m./Ad.OVA-i.v., or Ad.OVA-i.m./MVA.OVA-i.v. Mice were challenged 3-weeks after final immunization or 2-months after final immunization with OVA::Hep17 *P. berghei* spz. Data shown are representative of two independent experiments; Log-rank Mantel-Cox Test. p<0.0001(***); p<0.001(**); p<0.01(**); p<0.05(*); not significant (ns).

Fig. 5: Prime and target approach sterilely protects mice against challenge with transgenic spz expressing prominent clinical candidate *P. falciparum* vaccine antigens

BALB/c mice (n=6/group) were primed i.m. with ChAd63 viral vectors expressing the following antigens (A) ME-TRAP, (B) *Pf*LSA1 or (C) *Pf*LSAP2. Mice were subsequently vaccinated with either MVA-i.m. (ChAd63-i.m./MVA-i.m.), or ChAd63 i.v. (ChAd63-i.m./ChAd63-i.v.) each expressing the respective cognate antigen. For the ME-TRAP vaccinated group (A) an additional group of BALB/c mice (n=6/group) received ChAd63.ME-TRAP-i.m. followed by Np.Pb9-i.v. (ChAd63-i.m./Np.Pb9-i.v.). (D) CD-1 mice (n=6/group) were primed with ChAd63 viral vectors expressing *Pf*TRAP i.m. Mice received a subsequent immunization of either MVA i.m., or ChAd63 i.v. vectors each expressing *Pf*TRAP antigen. Protective efficacy of immunization was determined after challenge with (A) WT or (B-D) Tg *P. berghei* spz expressing the cognate *P. falciparum* antigen. Data shown are representative of two independent experiments. Statistical comparison (Log-rank Mantel-Cox Test) between ChAd63-i.m./ChAd63-i.v. and ChAd63-i.m./MVA-i.m. vaccinations: p<0.05(*); p<0.01(**).

Fig. 6: Safety and immunogenicity data following ChAd63.ME-TRAP i.v. vaccination in healthy human volunteers

(A) Following vaccination with ChAd63.ME-TRAP by intravenous peripheral cannula, safety was assessed by active and passive collection of local and systemic adverse events. The percentage of volunteers with local (left panel) and systemic (right panel) AE are shown for all tested dose groups: Group 1, 5x10⁸ vp; Group 2, 5x10⁹ vp; Group 3, 5x10¹⁰ vp (all n = 3). (B) Median with interquartile range of *ex-vivo* IFN γ ELISPOT responses to ME-TRAP peptides for each dose group from volunteers' PBMCs over time, measured in spot-forming cells (SFCs) per 10⁶ peripheral blood mononuclear cells (PBMCs). (C) Individual ELISPOT responses for each participant at day 14 after immunization, compared with data from a previous study with the same vaccine construct and dose, administered intramuscularly (54). Of note: subject in Group 3 (marked in red) was incubating mumps at time of immunization.

Figure 1

Ad.OVA-i.m./Np.OVA-i.v. ■
Ad.OVA-i.m./Np.OVA-i.m. ▲
Ad.OVA-i.m. □
naive ○

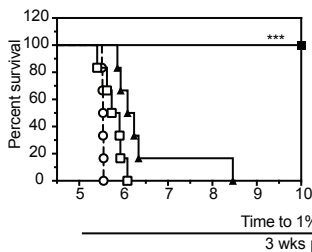
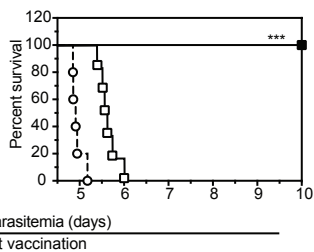
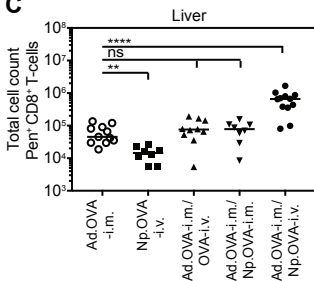
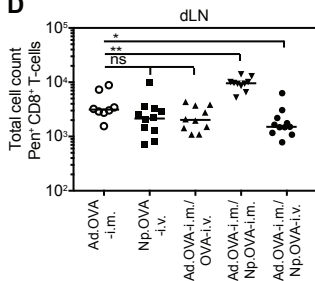
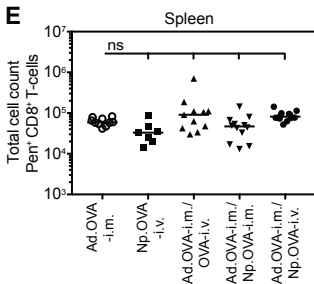
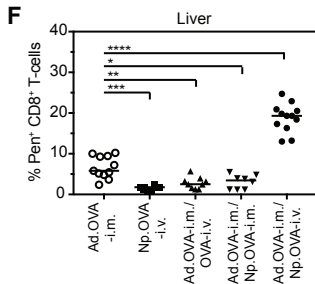
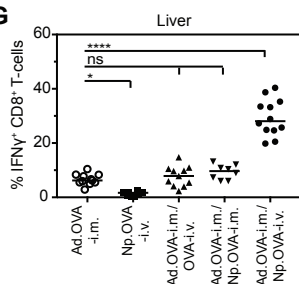
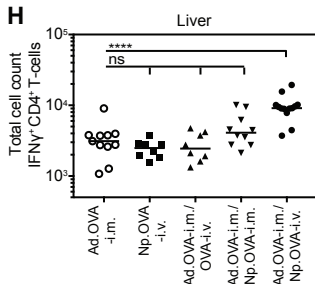
A**B****C****D****E****F****G****H**

Figure 2

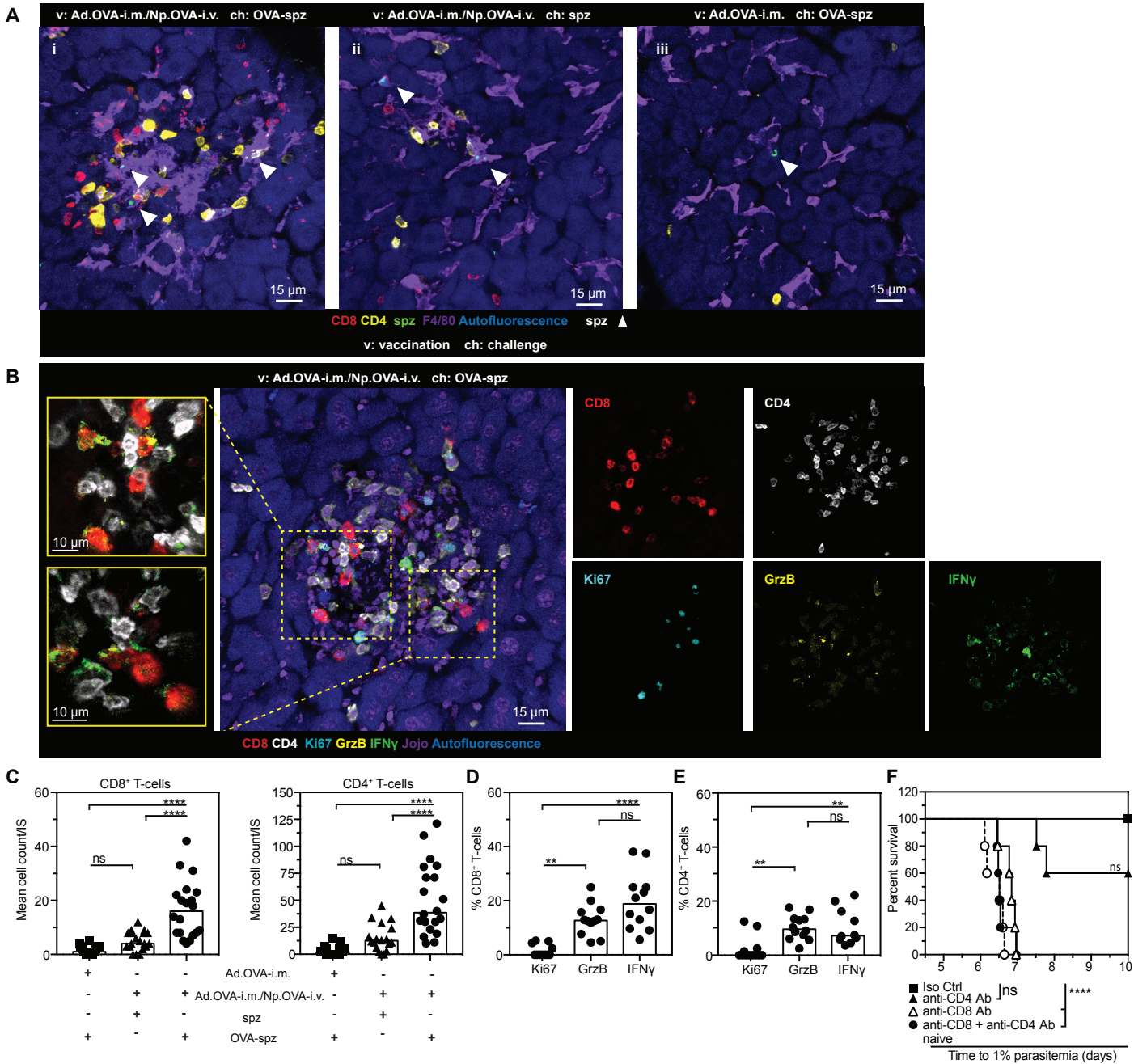


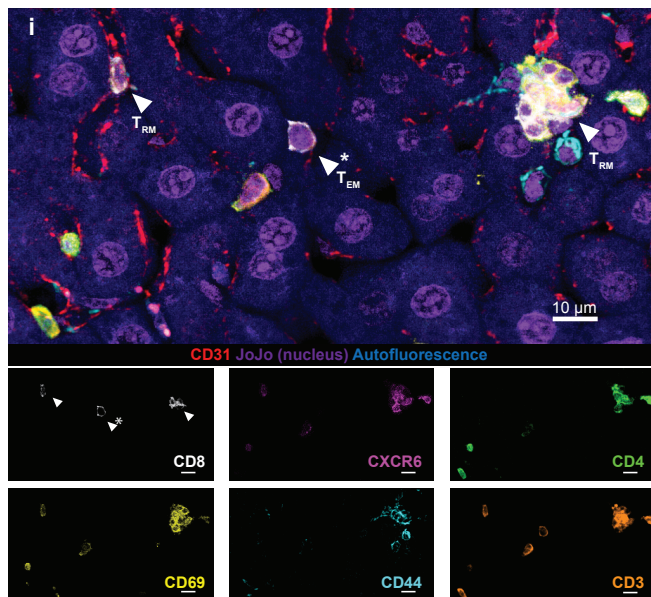
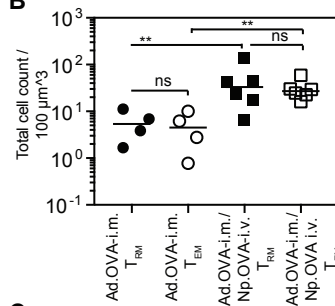
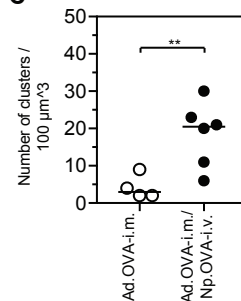
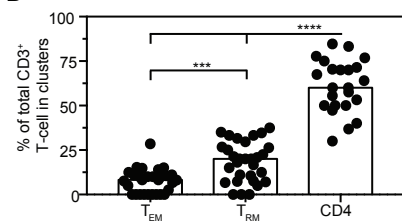
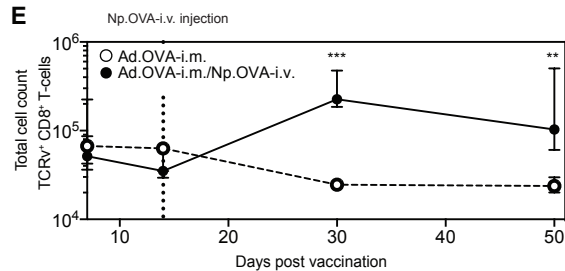
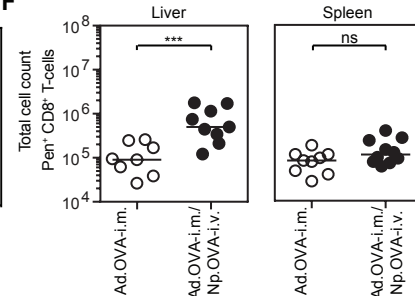
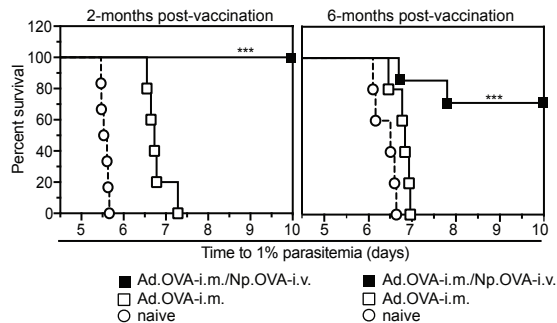
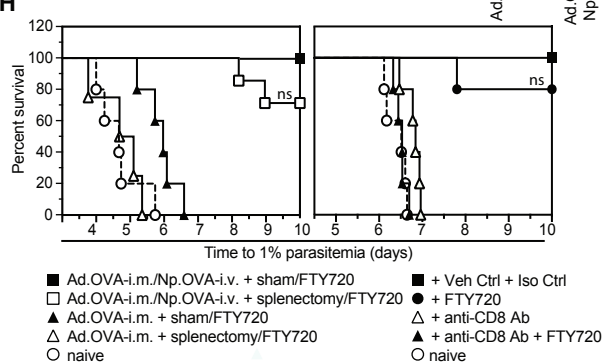
Figure 3**A****B****C****D****E****F****G****H**

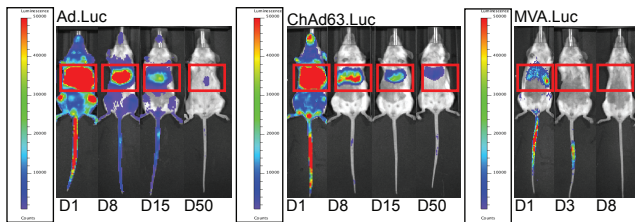
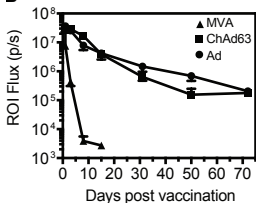
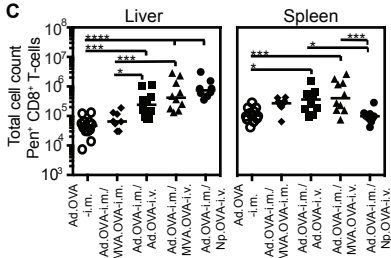
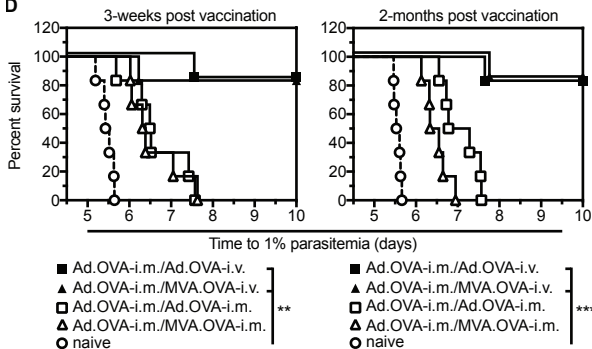
Figure 4**A****B****C****D**

Figure 5

ChAd63-i.m./Np.Pb9-i.v. ●
ChAd63-i.m./ChAd63-i.v. ■
ChAd63-i.m./MVA-i.m. △
naive ○

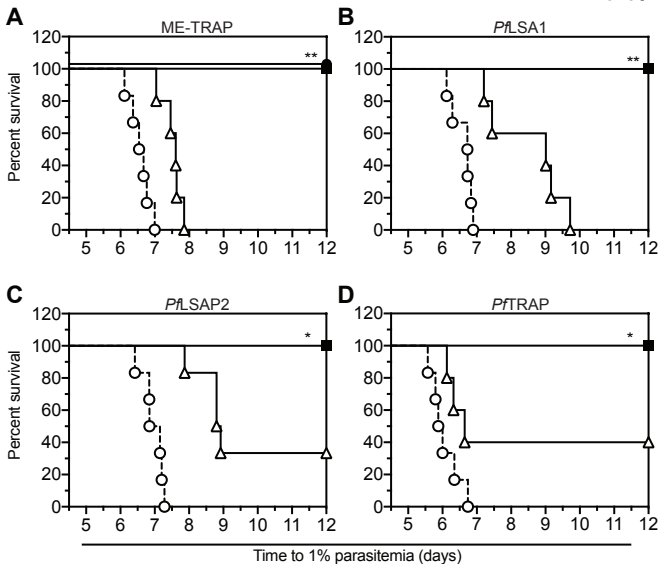
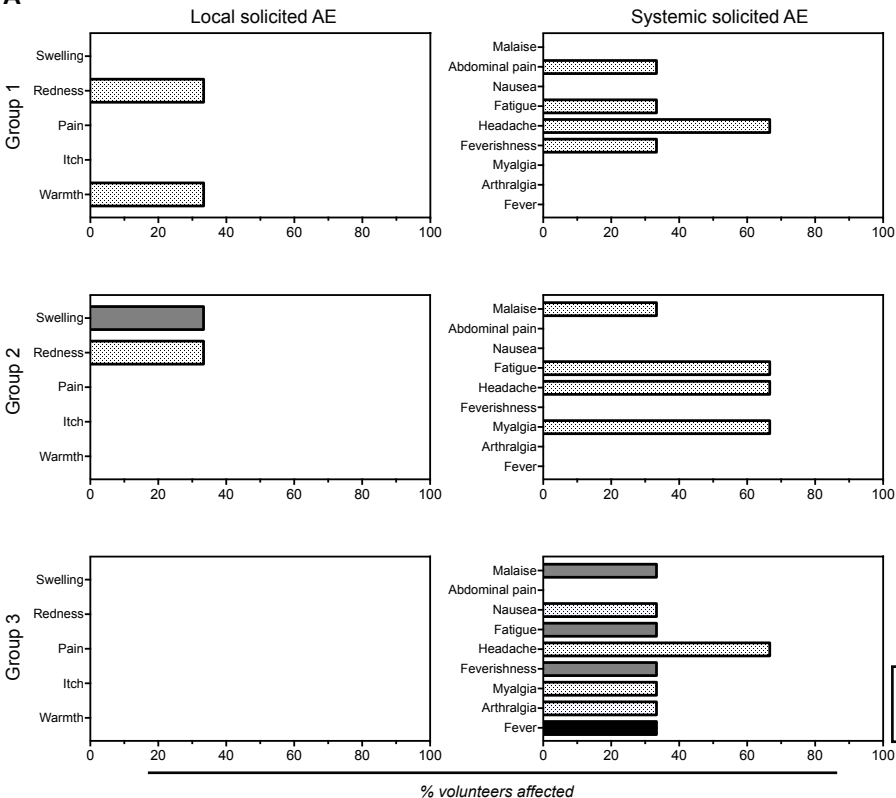
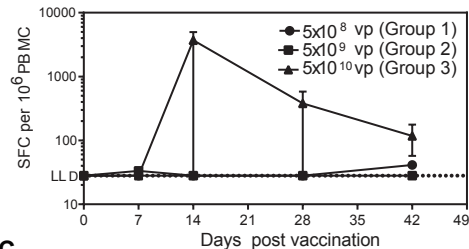


Figure 6**A****B****C**