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Mixed Vector Immunization With Recombinant Adenovirus and MVA Can Improve Vaccine Efficacy While Decreasing Antivector Immunity

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Substantial protection can be provided against the pre-erythrocytic stages of malaria by vaccination first with an adenoviral and then with an modified vaccinia virus Ankara (MVA) poxviral vector encoding the same ME.TRAP transgene. We investigated whether the two vaccine components adenovirus (Ad) and MVA could be coinjected as a mixture to enhance protection against malaria. A single-shot mixture at specific ratios of Ad and MVA (Ad+MVA) enhanced $CD8^+$ T cell-dependant protection of mice against challenge with *Plasmodium berghei*. Moreover, the degree of protection could be enhanced after homologous boosting with the same Ad+MVA mixture to levels comparable with classic heterologous Ad prime-MVA boost regimes. The mixture increased transgene-specific responses while decreasing the $CD8^+$ T cell antivector immunity compared to each vector used alone, particularly against the MVA backbone. Mixed vector immunization led to increased early circulating interferon-γ (IFN-γ) response levels and altered transcriptional microarray profiles. Furthermore, we found that sequential immunizations with the Ad+MVA mixture led to consistent boosting of the transgene-specific $CD8^+$ response for up to three mixture immunizations, whereas each vector used alone elicited progressively lower responses. Our findings offer the possibility of simplifying the deployment of viral vectors as a single mixture product rather than in heterologous prime-boost regimens.

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INTRODUCTION

Genetically modified human adenoviruses (Ad) and poxviruses are leading vaccine platforms in the development of prophylactic vaccines against infection by many pathogens, in particular where T-cell responses are protective (*e.g.*, malaria, HIV, hepatitis, and influenza viruses).[1] Unfortunately, the concomitant induction of vector-specific immune responses can result in a lack of prolonged expression of newly delivered genes upon readministration of the same vector after short time intervals.[2] This has prompted the development of new viral vector platforms or alternative serotypes of Ad, in particular of chimpanzee origin, which would allow repeated use of different vectors for either the same disease (as a boost), or for another disease in the same population.[1] In practical terms however, repeated boosting with the same vector could greatly facilitate the potential deployment of a vaccine, providing less expensive manufacture and administration compared with heterologous prime-boost immunization which requires different vectors encoding the same transgene to de be administered at different time points. In addition, there is still a need to improve the protective efficacy achieved by Ad- and poxvirus-based vaccines. In the context of malaria, protection against liver-stage relies on the induction of high frequencies of antigen-specific $CD8^+$ T cells producing interferon-γ (IFN-γ).[3] T-cell responses can effectively be induced by immunization with viral vectors such as fowlpox 9, modified vaccinia virus Ankara (MVA), and Ad.[4–7] However, we have previously shown that a single administration of Ad and poxviral vectors provides suboptimal protection to animals against a malaria sporozoite challenge, while prime-boost regimens using adenoviral vectors and MVA encoding the pre-erythrocytic ME.TRAP transgene enhanced both short- and long-term sterile protection against malaria.[8] These data highlighted the ability of optimized viral vector prime-boost regimens to generate more protective and sustained $CD8^+$ T-cell responses. Nevertheless, the levels of efficacy attained in clinical trials with Ad prime—MVA boost vaccine regimes encoding ME.TRAP are probably still insufficient for deployment of this single antigen vaccine on its own.[9] Therefore, increasing Ad and MVA vaccine-induced immunogenicity and efficacy remains a major goal.

In this context, we have previously observed that MVA can act as an adjuvant for a coadministered protein.[10] We demonstrated that repeated immunizations with recombinant or nonrecombinant MVA mixed with recombinant hepatitis B surface antigen induced higher titers of antibodies compared to immunization with either antigen alone or to formulations of the alum-adjuvanted Engerix-B vaccine (GlaxoSmithKline, Middlesex, UK). The poxviruses NYVAC, fowlpox, and ALVAC, and to a lesser extent, Ad, also displayed similar adjuvant properties when used in combination with recombinant hepatitis B surface antigen. In addition, we

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have also demonstrated that physically mixing a protein vaccine against murine malaria, CV-1866, with fowlpox 9 or MVA and then immunizing with the resultant combinations in a prime-boost regimen induced both cellular and humoral immunity and afforded substantially higher levels of protection than either the protein or poxviral vaccine alone. Therefore, combinations of partially effective vaccines may offer a more rapid route to achieving high efficacy than individual vaccine strategies.[11]

In this study, we immunized mice with either simian or human Ad vectors mixed with MVA (Ad+MVA) coding for the pre-erythrocytic-stage malaria antigen ME.TRAP, a transgene that has been used in clinical trials, containing the TRAP antigen from *Plasmodium falciparum* fused in-frame to a multiepitope (ME) string with multiple *P. falciparum* B-cell and T-cell epitopes, including the BALB/c H-2K^d-restricted epitope from *Plasmodium berghei* known as Pb9 (SYIPSAEKI).[12] We observed that specific ratios of Ad+MVA mixture enhanced CD8$^+$-dependant protection of mice against a stringent sporozoite challenge with *P. berghei*, after single-shot and homologous prime-boost regimes, and report initial analyses of the underlying mechanisms.

RESULTS

Mixed Ad+MVA vector immunization enhances CD8$^+$ T cell-mediated protection as compared to each vector alone

We explored whether the immunization with Ad and MVA mixed in the same formulation could enhance the protective efficacy over a single vector immunization. We used a mouse malaria model that relies on the generation of antigen-specific CD8$^+$ cells to mediate protection,[13] to this end, mice were vaccinated in the ear pinna with increasing doses of either AdC9 chimpanzee Ad or MVA alone, or with Ad+MVA mixtures, and challenged the mice two weeks later with *P. berghei* sporozoites (short-term protection). We noted that intermediate doses of Ad [5 × 10^9 viral particles (vp)] and MVA [0.1–1 × 10^6 plaque-forming unit (pfu)] appeared to elicit higher protection against the sporozoite challenge (55.6–61.3%, **Figure 1a**). Interestingly, the effect was dose specific and neither high nor low doses of mixed Ad+MVA elicited good protective levels (typically 0–33%, n = 6) (**Figure 1a**). Two vector mixtures at intermediate doses of AdC9 (5 × 10^9 vp) and MVA (1 × 10^5 and 1 × 10^6) were the most protective and the optimal dose of MVA (1 × 10^6 pfu) was chosen for the following experiments based on protective efficacy and immunogenicity, which elicited the highest frequencies of CD8$^+$IFN-γ$^+$ with increased levels of IFN-γ on a *per cell* basis (integrated median fluorescence intensity) and enhanced multifunctionality (**Figure 1b–d**).

To further extend our observations to other adenoviral serotypes and determine that any effect of a vector mixture immunization is not limited to a specific vector (v.gr. AdC9), we investigated this result in more detail using the clinically deployed adenoviral serotype, ChAd63 administered intramuscularly (**Figure 2**). The mixed ChAd63+MVA regime induced complete short-term sterile protection in two independent experiments while ChAd63 alone protected only 33% of the mice [hazard ratio of 17.73 with a 95% confidence interval (CI) (3.365–93.38) P = 0.0007], and MVA 0% [hazard ratio 46.22, 95% CI (0.001892–0.1076) P < 0.0001] (**Figure 2a**).

To investigate further the effect of the partially protective ChAd63 regime relative to the fully protective mixture regime, we used luciferase transgenic *P. berghei* parasites to quantify the parasite burden in the liver using an *in vivo* imaging system, in what we believe to be the first report of a malaria vaccine efficacy study using an *in vivo* imaging system method. We observed, in agreement with the results obtained from the parasite counts in blood, that the parasite burden in the liver appeared to be lower in the ChAd63+MVA group than in the ChAd63 or naive groups, although the differences were not quite statistically significant [mean for ChAd63 = 235,850 relative light unit, ChAd63+MVA = 122,367 relative light unit, 95% CI (−10,197 to 237,164), P = 0.06 by a t-test] (**Figure 2b,c**). Altogether, these results demonstrated a superior ability of vector mixtures to reduce the hepatic parasite burden and an ability to induce high levels of sterile protection against stringent liver-stage malaria challenges as compared to each vector alone.

Protection by Ad+MVA mixed vector vaccination can be enhanced in prime-boost regimes

We have previously reported that a single immunization with adenoviral vectors can induce short-term protection,[13] which can be enhanced upon vaccination with a heterologous viral vector expressing the same transgene.[8] We therefore assessed the protective ability of the Ad+MVA regime both shortly and long after a single vaccination and compared this to the best regime identified so far, Ad prime-MVA boost. For this experiment, BALB/c mice (n = 6 per group) were primed with AdC9, MVA or a mixture of both vectors (AdC9+MVA) and an additional homologous or heterologous vaccination was administered at week 8. For the heterologous prime-boost regime using the Ad+MVA mixture, we used AdC7 as an alternative serotype for the boost with the aim to determine if the Ad+MVA regimes require the use of heterologous vaccination to enhance efficacy and immunogenicity. Sporozoite challenge was administered 2 (short-term) or 8 (long-term) weeks after receiving the last vaccination (a study design we have previously described).[8] Another group was immunized once only with the AdC9+MVA mixture at the time of the boost and challenged 2 or 8 weeks postinjection along with the other groups (**Figure 3a**). Short-term protection, 2 weeks after the last vaccination, revealed that a single dose of Ad+MVA mixture elicited similar protective levels as the AdC9-MVA prime-boost (43%, **Figure 3b**). In addition, protection was even higher than AdC9-MVA shortly after mice were vaccinated twice with the homologous mixture AdC9+MVA—AdC9+MVA [86% protection, hazard ratio 3.638, 95% CI (0.4627–28.6) P = 0.22]. Sterile protection was induced using the heterologous mixture AdC9+MVA—AdC7+MVA [100%, hazard ratio 9.461, 95% CI (0.9159–97.72) P = 0.059 when compared to AdC9-MVA] (**Figure 3b**).

In a sporozoite challenge performed 8 weeks after last injection (long-term protection), a single vaccination with AdC9+MVA mixture did not induce any protection. However, a homologous AdC9+MVA prime—boost performed similarly to the heterologous AdC9 prime—MVA boost (71 versus 57%, respectively, P = 0.62, **Figure 3c**). Long-term complete sterile protection was achieved only in the group immunized twice with mixture vaccines (AdC9+MVA—AdC7+MVA hazard

a

Adenovirus dose/mouse ($\times 10^9$ vp)	MVA dose/mouse ($\times 10^5$ pfu)	Sterile protection against a challenge with 1,000 Sporozoites P. berghei		Statistical significance *P* value (vaccinated versus naive)
		Survived/challenged	%	
0	0	0/33	0	0
0	1	0/6	0	$P = 1.000$
0	10	0/6	0	$P = 1.000$
0	100	0/6	0	$P = 1.000$
1	0	1/6	16.7	$P = 0.019$
1	1	0/6	0	$P = 1.000$
1	10	1/6	16.7	$P = 0.019$
1	100	0/6	0	$P = 1.000$
5	0	3/18	16.7	$P = 0.017$
5	1	10/18	55.6	$P < 0.0001$
5	10	19/31	61.3	$P < 0.0001$
5	100	3/18	16.7	$P < 0.017$
10	0	2/6	33.3	$P < 0.0008$
10	1	2/6	33.3	$P < 0.0008$
10	10	3/6	50	$P < 0.0001$
10	100	2/6	33.3	$P < 0.0001$

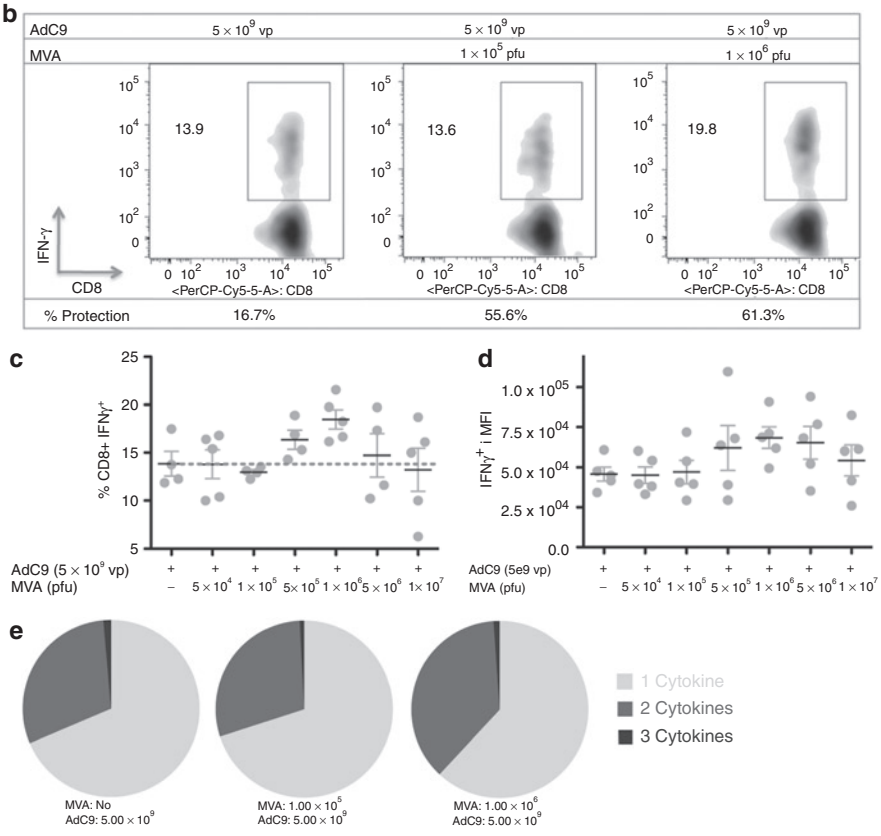


Figure 1 Enhanced protection by an adenovirus (AdC9) and modified vaccinia virus Ankara (MVA) vector mixture (Ad+MVA) vaccine intradermally as compared to each vector alone in a *Plasmodium berghei* malaria pre-erythrocytic challenge. (**a**) Dose-escalation study to assess the protective efficacy of various ratios of Ad+MVA vaccine. BALB/c mice received a single immunization with viral vectors alone or as a mixture at increasing doses and modified ratios of viruses. Two weeks later, all mice were challenged by i.v. injection of 1,000 *Plasmodium berghei* sporozoites and screened from day 5 to 20 for presence of parasites in blood using Giemsa-stained smears. Outcome was measured as presence or absence (sterile protection) of parasites in blood. Experiments were performed once with the exception of the MVA group alone and the middle dose of adenovirus (5×10^9 viral particles (vp)) that elicited better protection for which these groups were assessed three to five times in independent experiments ($n = 6$–7/group). Experiments yielded similar results and data was pooled. Statistical analysis was performed by comparing every immunized group to the naive animals. (**b**) Comparison of the immune responses in representative mice that were immunized with AdC9 or with a mixture of AdC9 containing MVA at the two most protective doses [1×10^5 and 1×10^6 plaque-forming unit (pfu)] to determine the most immunogenic composition. (**c**) Interferon (IFN)-γ antigen-specific CD8$^+$ responses elicited by immunization with viral vectors alone (AdC9) or as a mixture (AdC9+MVA) using increasing doses of MVA. (**d**) Integrated median fluorescence intensity (iMFI) (%IFN-γ × MFI) in the same experiment as (c) and (**e**) Enhancement of functionality (2+) of antigen-specific CD8+ cells by Ad+MVA immunization. Functionality was assessed by expression of 1 (1+), 2 (2+). or 3 cytokines (3+) upon stimulation with Pb9 peptide and staining with anti-CD8, IFN-γ, tumor necrosis factor (TNF)-α and interleukin (IL)-2.

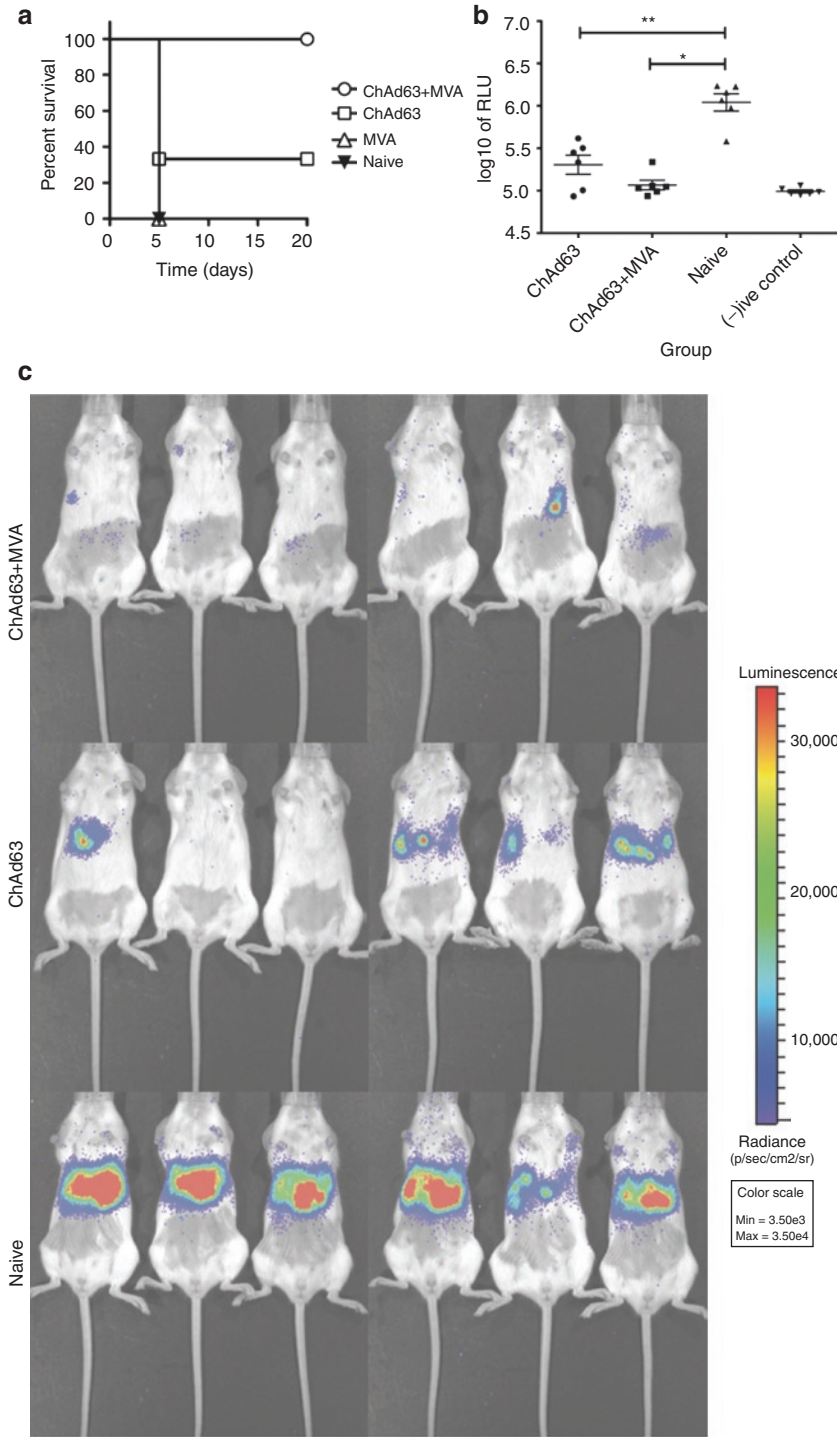


Figure 2 Enhanced protection by an adenovirus (ChAd63) and modified vaccinia virus Ankara (MVA) vector mixture (Ad+MVA) vaccine intramuscularly in a Plasmodium berghei malaria pre-erythrocytic challenge. BALB/c mice ($n = 12$) were immunized intramuscularly with ChAd63 [5×10^9 viral particles (vp)], MVA [1×10^6 plaque-forming unit (pfu)] and ChAd63+MVA (same concentrations) all expressing ME.TRAP. (**a**) Mice were challenged with 1,000 transgenic luc *P. berghei* sporozoites and blood was screened for the presence of parasites from day 5 to 20 after challenge. The graph shows results from two independent challenges ($n = 6$ for each one). (**b**) Quantitative analysis of luciferase signal expressed as photons per second of imaging time after a challenge using 1,000 luciferase transgenic *P. berghei* parasites ($n = 6$). (**c**) Overlay of grayscale photo and luciferase bioluminescence image.

ratio compared to AdC9-MVA = 0.106, 95% CI (0.01–1.092) $P = 0.059$; **Figure 3c**). These results indicate that homologous Ad+MVA prime-boost induces protection against a sporozoite malaria challenge similar to the heterologous Ad-MVA regime. Nevertheless, the heterologous mixture regimes composed by AdC9+MVA followed by AdC7+MVA induced the most robust protection, which protected all mice at both short- and long-term after vaccination.

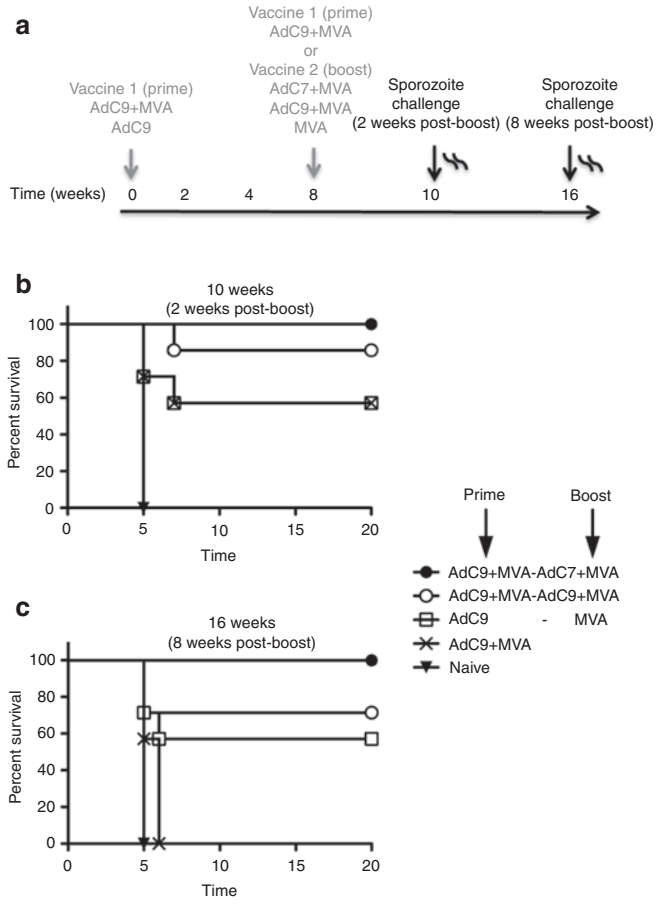


Figure 3 Protective efficacy elicited by prime-boost vaccination with Ad+MVA vector mixtures in a Plasmodium berghei sporozoite challenge. (**a**) Groups of BALB/c mice ($n = 6$) were vaccinated with AdC9 or a vector mixture AdC9+MVA-expressing ME.TRAP. Mice were boosted 8 weeks later with modified vaccinia virus Ankara (MVA) (heterologous), AdC7+MVA (heterologous) or AdC9+MVA (homologous) expressing ME.TRAP while a group was primed with AdC9+MVA at concentrations described in **Figure 2**. All groups of mice were challenged by intravenous injection of 1,000 *P. berghei* sporozoites at weeks 2 and 8 post-boost. (**b**) Short-term protective efficacy elicited by homologous or heterologous prime-boost regimes. Mice were challenged two weeks after receiving the booster vaccination (week 10). (**c**) Long-term protective efficacy elicited by prime-boost regimes. Mice were challenged on week 8 after the second vaccination (week 16). Graphs show results of a single experiment with a challenge at two different time points.

Immunization with AdC9+MVA permits multiple readministration of the same formulation (homologous prime-boost)

We observed in our previous results that Ad+MVA vector mixture immunization could induce prolonged protection against pre-erythrocytic malaria. We next assessed the effect of repeated administration of the same mixture on $CD8^+$ T-cell responses to the protective Pb9 epitope (**Figure 4a**). A single immunization with AdC9+MVA induced similar frequencies of IFN-γ-secreting $CD8^+$ T cells [mean 18,708 spot-forming units/10^6 peripheral blood mononuclear cell (PBMC)] to AdC9 (mean 15,414 spot-forming units/10^6 PBMC) which were significantly higher than frequencies induced by MVA (1,158 spot-forming units/10^6 PBMC, $P < 0.001$). A second homologous immunization with AdC9+MVA significantly enhanced the $CD8^+IFN\text{-}\gamma^+$ frequencies (mean 80,214/10^6 PBMC) compared to AdC9 (mean 42,671/10^6 PBMC, $P < 0.05$) and MVA (mean 23,983 spot-forming units/10^6 PBMC $P < 0.001$). Surprisingly, a third homologous immunization with AdC9+MVA further increased the $CD8^+$ responses to 180,847/10^6 PBMC (**Figure 4a**). A fourth homologous vaccination was still able to boost the memory $CD8^+$ responses but they did not surpass the frequencies that were reached in the peak following the third immunization. While it is well known that pre-exposure to Ad (naturally or through a vaccine) can markedly impair the immunogenicity of a subsequent Ad vector,[2] our results suggest that this may not be the case with the Ad+MVA mixture. We further assessed if the vector mixture immunization requires expression of the same transgene by both viruses or whether MVA can adjuvant the Ad-induced responses despite expressing an irrelevant transgene. Optimal immunogenicity was elicited when both, Ad and MVA expressed the same transgene (ME.TRAP). Nevertheless, $CD8^+$ responses where significantly lower when MVA expressed an irrelevant transgene (lacz) (**Figure 4b**). We further confirmed this observation in an additional independent experiment where Ad+MVA vector mixtures expressed different transgenes (ME.TRAP from *P. falciparum* and Ag 85A from *Mycobacterium tuberculosis*) (**Figure 4c–e**). Again, $CD8^+$ responses were optimal when both vectors expressed the same transgene (AdH5+MVA) ME.TRAP (**Figure 4c**) or (AdH5+MVA) Ag85A (**Figure 4d**). Availability of an immunodominant $CD4^+$ epitope in 85A revealed that frequency of these cells is enhanced when both vectors express the same Ag 85A transgene (**Figure 4e**).

Mechanisms behind the Ad+MVA mixed vector immunization: kinetics of transgene expression and antivector immunity

We sought to understand how the mixture regime might achieve the potent protective effects demonstrated. The action of the two viruses used differs markedly. For example, studying the *in vitro* kinetics of the expression of a GFP transgene in permissive cells after Ad or MVA infection, MVA induced a rapid transgene expression that peaked at 5–6 hours, while Ad transgene expression reached a maximum after 18–20 hours (**Figure 5a**). Induction of T cells *in vivo* also differs, perhaps due to different antigen expression kinetics: transgene-specific $CD8^+$ T-cells peak at 1 week after MVA vaccination, while adenoviral vectors induce a delayed $CD8^+$ response that peaks at 3 weeks post-prime.[14]

A prime-boost regime might be expected to enhance the T-cell immune responses against the shared antigen (here the transgene) at the expense of the nonshared antigens (here the viral vectors backbone).[15] Thus, in Ad+MVA mixed immunization, T cell responses to the ME.TRAP transgene might be expected to be privileged (dominant as well as subdominant responses) while the antivector T-cell immunity would decrease as compared to single vector immunization. Therefore, we assessed the transgene-specific responses against the dominant Pb9 epitope located in the N-terminal region of our transgene (the ME string) as well as the subdominant T-cell epitopes located in the C-terminal region, TRAP. We observed that Pb9 responses were higher after Ad+MVA mixture immunization as compared to single Ad and

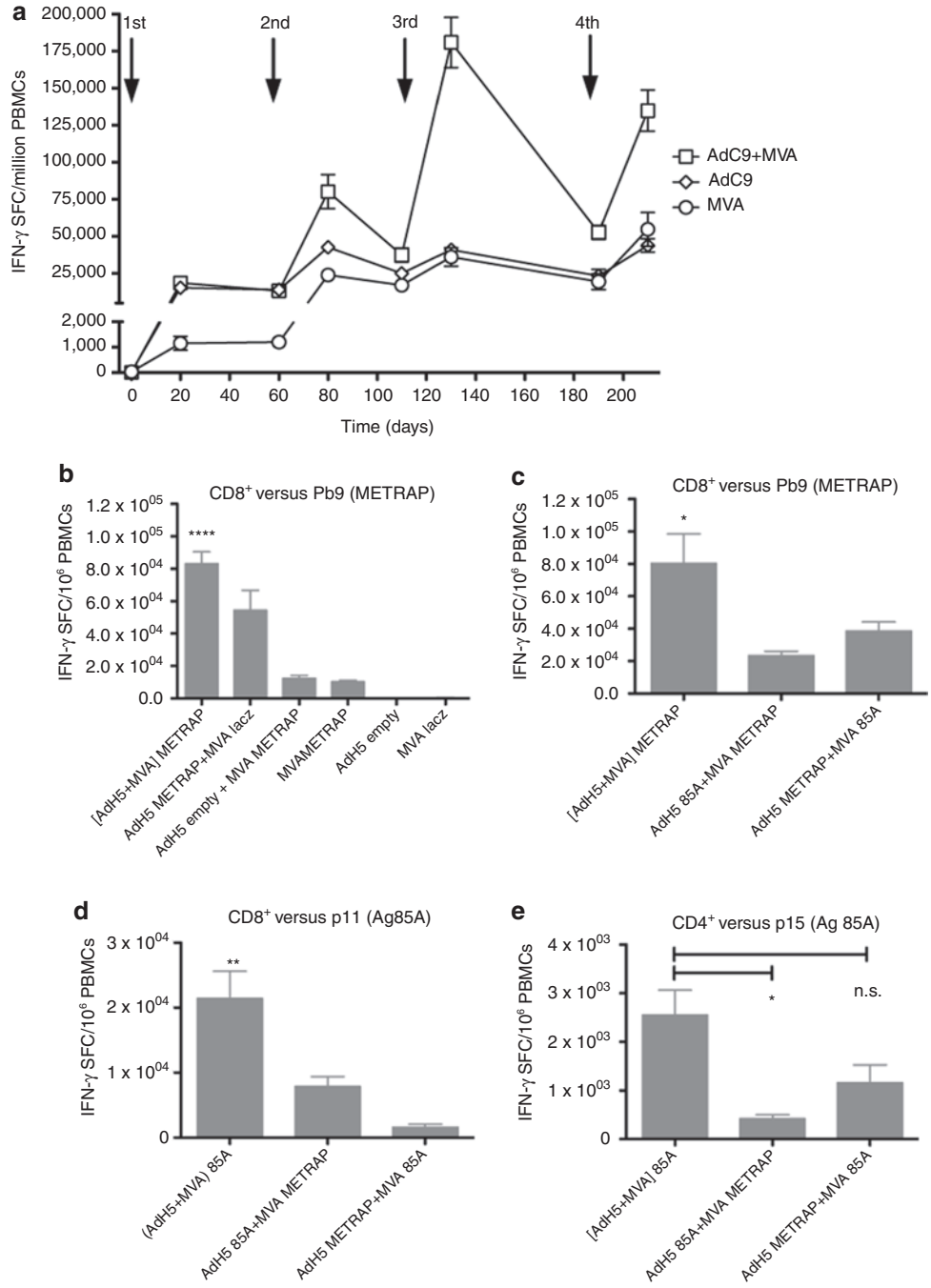


Figure 4 Ad+MVA vector mixtures can be injected repeatedly as homologous prime-boost regimes to enhance antigen-specific CD8$^+$ responses. (**a**) BALB/c mice ($n = 6$) received homologous immunizations using AdC9 ME.TRAP [5 × 10^9 viral particles (vp)/mouse], modified vaccinia virus Ankara (MVA) ME.TRAP (1 × 10^6 plaque-forming unit (pfu)/mouse) or a vector mixture containing AdC9+MVA-expressing ME.TRAP (5 × 10^9 vp and 1 × 10^6 pfu, respectively). CD8$^+$ responses against the H2 K^d-restricted immunodominant Pb9 peptide (ME string) were assessed by ELISPOT at various time points after immunization. Data shown corresponds to the mean and SEM for each group in a single experiment. Similar results were obtained in an additional experiment. (**b**) CD8$^+$ responses against the Pb9 epitope from ME.TRAP in BALB/c mice that received two homologous immunizations with an AdH5+MVA mixture expressing the same transgene (ME.TRAP), a different transgene (MVA lacz) or empty vector (AdH5empty). (**c**) CD8$^+$ responses against the Pb9 epitope from the ME.TRAP in mice immunized twice with an AdH5+MVA vector mixture expressing the same transgene (ME.TRAP) or different transgenes (Ag 85A from *M. tuberculosis*). (**d**) CD8 and (**e**) CD4 responses elicited against the Ag 85A by vector mixtures expressing homologous (Ag 85A) or heterologous (Ag 85A and ME.TRAP) transgenes. Results shown correspond to three independent experiments.

these were subsequently enhanced upon sequential homologous prime-boost regimens (**Figure 5b**).

In addition, both the breadth (number of epitopes recognized by any one mouse as well as number of mice responding to any one epitope) and magnitude (average IFN-γ SFC/million PBMC in responding mice) of the T-cell subdominant responses directed toward the TRAP transgene were increased when Ad+MVA vaccination was used when compared to each vector alone,

indicating that precursor frequencies and hierarchy of responses are modified by the use of vector mixtures (**Figure 5c**).

Ad+MVA mixture decreases CD8$^+$ T-cell responses to the MVA vector

We further investigated whether the mixture-induced increases in the transgene-specific response are accompanied by a decrease of vector-specific CD8$^+$ responses. To this end, we constructed an Ad vector expressing a dominant MVA CD8$^+$ epitope (E3, coded by the gene *E3L*) and primed groups of mice with AdHu5-E3L, or an Ad expressing a strong CD8$^+$ epitope Pb9 (AdHu5-TIP). Unprimed mice acted as controls. The mice were then boosted 8 weeks later with MVA-TIP (expressing the major MVA CD8$^+$ T-cell epitopes E3L and F2G, as well as Pb9) and the CD8$^+$ responses against the three epitopes Pb9 (H-2K^d), E3 (D^d), and F2G (L^d)[16] were quantified.

As expected, we observed that when mice were primed and boosted with the vectors encoding the same Pb9 transgene, strong responses were focused toward the shared Pb9 antigens at the expense of the nonshared MVA epitopes (E3 and F2G) (**Figure 5d**), which were decreased as compared to unprimed mice. When mice were primed with an Ad encoding *E3L* and then boosted with MVA-TIP, the response to the shared epitope E3 was strongly favored, at the expense of the Pb9 epitope (which was drastically decreased as compared to unprimed mice, **Figure 5d**) or the F2G epitope contained in MVA but absent in the Ad-*E3L* prime. These results confirm and extend to MVA the observations in Ad from Schirmbeck *et al.*[15] that CD8$^+$ responses to the vector and transgene compete with each other, and demonstrate as expected that indeed a prime—boost regime typically favors CD8$^+$ response to shared epitopes between prime and boost, whether it is in the transgene or in the vector backbone.

To further confirm decreased antivector immunity, we assessed the CD8$^+$ responses directed against both Ad and MVA in the vector mixture by using major CD8$^+$ epitopes described for each vector. Measuring anti-Ad immunity in an experiment required the use of AdH5 as epitopes in BALB/c mice have been described for this particular serotype.[15] For MVA these were F2G (L^d), E3 (D^d), and C6S (H-2K^d) (**Figure 5e**). We demonstrated that as soon as 1 week after immunization, anti-MVA CD8$^+$ T-cell responses were significantly lower in the Ad+MVA mixture than in the MVA groups for the three dominant epitopes (**Figure 5e**). Moreover, this decreased anti-MVA CD8$^+$ response was observed at all time points tested, 1 and 2 weeks after 1, 2, or even 3 injections (data not shown). The CD8$^+$ response to Ad however was only marginally decreased for the L^d-restricted epitope dbp7 (located in the DNA-binding protein), while no decrease was observed against the H-2K^d-restricted hex3 (hexon) epitope.[15] The latter may not be surprising, however, because the Ad preparation contains free hexon particles, detected by the immune system before any expression from the virus occurs.

Additionally, we confirmed that the antivector immunity decreases when both Ad and MVA are injected as a mixture by pre-exposing BALB/c mice to Ad, MVA or Ad+MVA expressing an irrelevant transgene (Ag 85A). Four weeks after the initial exposure to viral vectors, all groups were immunized with Ad+MVA expressing ME.TRAP and the transgene-specific Pb9 responses were assessed (**Figure 5f**). As compared to a control group (no pre-exposure followed by Ad+MVA, mean of 17,108 SFC/million PBMCs), Pb9-specific responses showed a nonsignificant trend to reduction in mice pre-exposed to MVA-85A (mean 14,294 SFC/million PBMCs. $P = 0.25$) and a significant reduction when pre-exposed to Ad 85A (mean 9,096 SFC/million PBMCs, $P = 0.009$). By contrast, Pb9 responses were not reduced in mice injected initially with an Ad+MVA Ag85A mixture (mean of 16,686 IFN-γ SFC/million PBMCs, $P = 0.88$). We also assessed the effect Ad+MVA mixed immunization on the generation of neutralizing antibodies against AdHu5 (**Figure 5g**). We observed that sera from mice immunized with Ad+MVA contained similar levels of neutralizing antibodies against the Ad vectors to those from immunized with Ad and similar titers of antibodies against the TRAP transgene in Ad and Ad+MVA immunized mice, both after priming (**Figure 5h**) and after four consecutive homologous immunizations (data not shown), indicating that the vector mixture reduces the anti-Ad immunity by decreasing the vector-specific CD8$^+$ responses rather than the antibodies directed toward the external parts of the Ad. Finally, we investigated whether both vectors could also be administered in separate sites (coadministration) to enhance immune responses in a similar way to the vector mixture (**Figure 5i**). We observed that coadministration of Ad and MVA (without physically mixing them) significantly enhanced the antigen-specific CD8$^+$ frequencies after a homologous prime-boost regime when compared to a single priming immunization (mean after prime = 9.0%; boost = 21.7%, 95% CI (−21.58 to −3.748), $P < 0.001$). Significant enhancement was also induced by the vector mixture (physically mixed and administered in the same site) in a homologous immunization (mean after prime = 9.7%; boost = 24.2%, 95% CI (−23.41 to −5.574) $P < 0.0001$). Increase in immunogenicity by homologous AdH5 (mean after prime = 11.2%; boost = 18.5%, 95% CI (−16.18 to 1.655)) or MVA (mean after prime = 2.2%; boost = 6.9%, 95% CI (−13.63 to 4.201)) was not significant.

Ad+MVA vaccination does not influence the CD8$^+$ T central memory responses

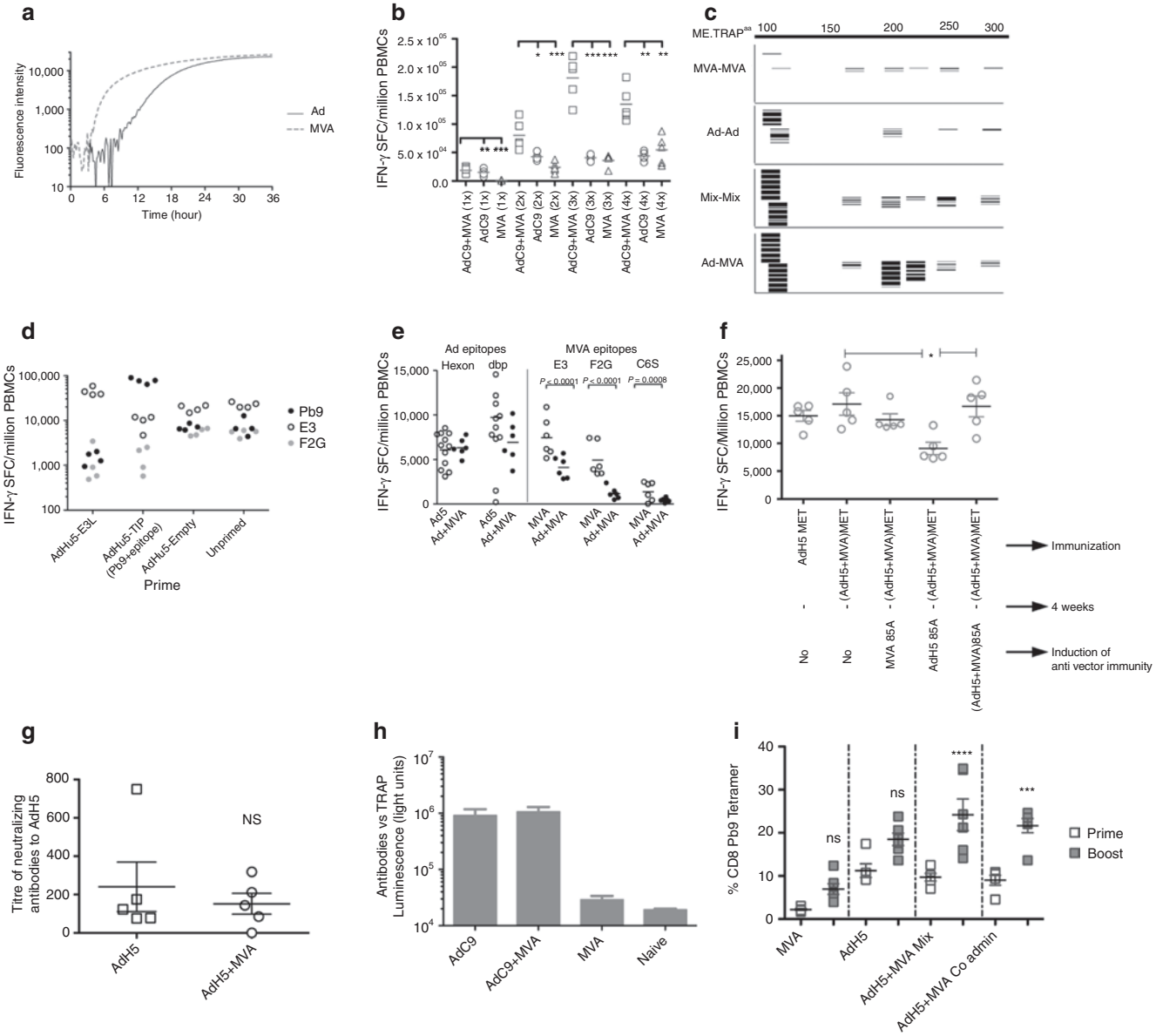
We further explored whether the enhanced proliferative ability of CD8$^+$ T cells elicited by homologous Ad+MVA prime-boost was related to a preferential T central memory (T_{CM}) phenotype induction. It has been shown that CD8$^+$ T_{CM} cells (CD62L$^+$CD127$^+$) have a high proliferative capacity, whereas T_{EM} (CD62L$^-$CD127$^+$) are more limited and no proliferation is possible by T_E (CD62L$^-$CD127$^-$) cells.[17] We have previously demonstrated that MVA induces a preferential and accelerated CD8$^+$ T_{CM} phenotype on antigen-specific cells, which contrasts with a marked $T_{E/EM}$ phenotype induced by Ad.[14] This led us to hypothesize that an Ad+MVA vaccine mixture could skew the $T_E/T_{EM}/T_{CM}$ population as compared to Ad alone.

We immunized BALB/c mice with MVA, AdH5 and AdH5+MVA regimes and assessed the CD8$^+$ memory phenotype of Pb9-specific cells 60 days later using a Pb9 tetramer and costaining with anti-CD62L and CD127. Our results indicated that the Ad5+MVA mixture does not enhance CD8+ T_{CM} but rather resemble the profile obtained with AdH5 (**Figure 6a**). Therefore the increased CD8$^+$ proliferation seen in Ad+MVA regimes

cannot be attributed to enhanced T_{CM} responses. Interestingly, in the same analysis we detected that there was a high proportion of $CD8^+$ T_{EM} responses elicited by Ad+MVA immunization, which was only significant when compared to MVA (mean of 82.1% for AdH5+MVA; and 64.43 for MVA; $P < 0.01$) (**Figure 6b**). This result was of particular interest as we have recently demonstrated that the T_{EM} phenotype mediates protection in a mouse malaria model.[14]

Effect of the Ad+MVA immunization on innate immune responses

We explored whether a vaccine consisting of a mixture of two viral vectors (Ad+MVA) modifies innate immunity, perhaps altering dendritic cell (DC) maturation. BALB/c mice were immunized with MVA ME.TRAP, the chimpanzee Ad AdC9 ME.TRAP, or a vector mixture containing AdC9+MVA as a single preparation. To determine directly the degree of *in vivo* DC activation, we assessed the upregulation of two canonical costimulatory molecules CD80 and CD86 in DCs ($CD11c^+$) from draining lymph nodes 18 hours after immunization (**Figure 7**). In agreement with a previous report, MVA alone was shown to induce moderate *in vivo* DC activation.[18] MVA induced elevated levels of CD86 expression that was comparable to 10 μg lipopolysaccharide however, there was no significant increase in CD80 expression between MVA stimulated and naive. Conversely, Ad and Ad+MVA groups were more immunostimulatory than 10 μg lipopolysaccharide and induced significantly higher CD80 and CD86 expression (**Figure 7a–c**). Results here demonstrate that Ad is more potent than MVA in stimulating innate immune response *in vivo*. Importantly, the addition of MVA to Ad does not dampen the DC stimulating properties of the latter. A further analysis of the innate responses was performed using a multiplexed bead-based immunoassay (BD cytometric bead array) to determine the serum concentration of the proinflammatory cytokines IFN-γ, MCP-1, tumor necrosis factor (TNF), interleukin (IL)-6, IL-10, and IL-12p70 24 hours after intradermal immunization. Mice vaccinated with the AdC9+MVA mixture



had significantly higher levels of circulating IFN-γ than AdC9 (mean of 40.8 pg/ml for AdC9+MVA, compared to 13.8 pg/ml for AdC9) and MVA (mean of 3.38 pg/ml, overall *P* value <0.0001) (**Figure 7d**). This threefold increase in circulating levels of IFN-γ was not due to an additive effect by both viruses present in the same vaccine as this would have resulted in only a 1.24-fold increase of the AdC9-induced IFN-γ (17.18 pg/ml, 13.8 pg/ml by Ad plus 3.38 pg/ml by MVA, would be the expected additive value as opposed to the 40.8 measured). Similarly, higher levels of MCP-1 were induced by AdC9+MVA as compared to the vectors alone but this was only significantly higher than MVA (mean of 107.8 pg/ml for AdC9+MVA, compared to 85.5 pg/ml for AdC9, and 41.3 pg/ml for MVA, $P < 0.0001$) (**Figure 7e**). Both AdC9 and AdC9+MVA vaccines induced the highest levels of circulating TNF but no significant differences were detected for any of the regimes (**Figure 7f**). Monitoring of additional cytokines showed that neither IL-6, IL-12p70, nor IL-10 were induced preferentially by any vaccine (data not shown).

Microarray analysis of gene expression following vaccination with viral vectors

To further investigate potential innate pathways underlying the observed effects of vaccination with the Ad+MVA mixture, we carried out a gene expression analysis using whole mouse genome microarrays (GEO accession number GSE30564). Multiple groups of 4 mice were vaccinated intradermally into both ears with either Ad, MVA or Ad+MVA mixture. Ear biopsies (vaccination site) or draining cervical lymph nodes were collected at several time points (6 hours and 24 hours for ear biopsies; 9 hours, 24 hours, and 72 hours for lymph nodes). Differential gene expression assessment was carried out between each treatment/time-point and the corresponding samples from nonvaccinated animals (**Figure 8**). At the site of vaccination, we observed a striking increase in the number of differentially expressed genes with time: in a per-gene analysis, 6 hours after vaccination fewer than 50 genes were found to be significantly ($P < 0.05$) up- or downregulated in the biopsies from mice vaccinated with Ad or MVA and no differentially regulated genes were found in the mice vaccinated with the mixture (**Figure 8a**) suggesting that their coadministration impacted on their individual behavior.

At 24 hours postvaccination, over a hundred genes were significantly differentially expressed as a result of immunization with Ad, and several-fold more in the mice vaccinated with MVA and the mixture (**Figure 8a**). Top 20 genes upregulated at the site of vaccination in response to the Ad+MVA mixture were also upregulated by individual Ad or MVA vaccination and the majority were immune response-related (*e.g.*, chemokine ligands, lipocalin), or IFN type I and II induced proteins such as guanylate-binding protein, IFN-γ-induced GTPase, IFN-induced protein with tetratricopeptide repeats. In ear biopsy samples from animals injected with MVA or the Ad+MVA mixture, but not with Ad alone, the two highest upregulated genes were keratin and stefin, which are involved in epithelial proliferation and development. A heat-map representation of all genes up- and downregulated in the ear biopsies by at least threefold and reaching the adjusted *P* value of 0.01 illustrates the similarities of the gene expression patterns induced by the Ad+MVA mixed vaccine to one or both of the Ad and MVA vaccines. The heat-map revealed that only a few genes are differentially upregulated by the mixture compared to the individual vectors, for instance, S100a8 at 6 hours and Fcrl3, Ly6c, Psmb10, and Usp18 at 24 hours.

The earliest time-point analyzed for the lymph nodes, 9 hours postvaccination, showed a few thousand significantly up-and

Figure 5 Effect of the Ad+MVA vector mixture on the shared and nonshared transgenic protein and antivector immunity. (**a**) Kinetics of *in vitro* infection by ChAd63-GFP (Ad) and MVA-GFP vectors. Confluent 293 cells (Ad) or BHK cells (modified vaccinia virus Ankara (MVA)) were infected with one infectious unit (Ad) or plaque-forming unit (MVA) per cell of virus in duplicate in black-wall 96-well plates, which were immediately placed at 37 °C + 5%CO_2 in a BMG Fluostar fluorimeter for measurement of GFP fluorescence every 6 minutes for 36 hours. Lines indicate the mean of two duplicates. Random noise is apparent close to the limit of detection (<200 units). (**b**) Groups of BALB/c mice ($n = 6$) received homologous or heterologous prime-boost immunizations as described in **Figure 3a** and an analysis of the $CD8^+$ responses against the H-$2K^d$-restricted immunodominant Pb9 peptide (ME string transgene) were assessed over time by interferon (IFN)-γ ELISpot. (**c**) T-cell responses against the seven subdominant epitopes identified in the TRAP region of the ME.TRAP transgene. Graphs depict responses 2 weeks after second injection with Ad or MVA or Ad+MVA mixed. Each line represents an individual animal responding to the peptide [amino acid (aa) position indicated on top]. Six animals per group were assessed against all peptides, and the weight of the lines is proportional to the intensity of the response as follows: 0–100 SFC/million peripheral blood mononuclear cell (PBMC); 101–200; 201–300; 301–400; 401–500; 501–600, >601 SFC/million PBMCs. Statistical analysis was performed using Wilcoxon rank-sum test: Ad+MVA versus Ad, $P = 0.0003$, Ad+MVA *vs* MVA, $P = 0.0000$. (**d**) Prior immunization with adenovirus expressing immunodominant T cell antigens suppresses responses to other antigens in MVA-boosted mice. BALB/c mice were immunized intradermally (i.d.) with 10^9 vp of AdHu5 expressing TIP-an epitope string containing Pb9-; the vaccinia virus gene *E3L*; or lacking a transgenic open-reading frame (empty); or were not primed. Eight weeks later, all mice were boosted i.d. with 10^6 plaque-forming unit (pfu) of MVA-METRAP and responses to the immunodominant Pb9, E3 and F2(G) $CD8^+$ T cell epitopes were measured by interferon (IFN)-γ ELIspot using stimulation with the relevant synthetic peptides. (**e**) Antivector $CD8^+$ T-cell responses directed against the hexon and DNA binding protein of the adenovirus and the three major $CD8^+$ MVA epitopes (E3, F2G, and C6S). Graph depicts responses 1 week immunizations with Ad or MVA alone (empty circles) or Ad+MVA mixed (black circles), except for dbp for which responses at 2 weeks are shown. Each dot represents an individual animal and horizontal lines represent the geometric mean. Transgene is referred to as *E3L*. The expressed protein is E3. (**f**) Effect of the Ad+MVA vaccine on the pre-existing immunity and transgene-specific $CD8^+$ responses. BALB/c mice ($n = 5$) were injected with Ad, MVA, or Ad+MVA expressing an unrelated transgene (Ad 85A from *M. tuberculosis*) and then immunized 4 weeks later with the Ad+MVA expressing the Pb9 epitope. Pb9-specific $CD8^+$ responses were measured 2 weeks after the final Ad+MVA immunization. Circles represent individual mice and lines the mean and SEM. (**g**) Effect of the Ad+MVA vector mixture on the induction of neutralizing antibodies against the adenoviral vector. BALB/c mice were immunized with AdH5 or AdH5+MVA ME-TRAP and sera was obtained 2 weeks later. Neutralizing antibody titres were calculated by incubating the sera with AdH5 expressing a reporter gene and calculating the inhibition of infection in HEK293 permissive cells. (**h**) Quantification of antibodies against the TRAP transgene. BALB/c mice ($n = 6$) were immunized with AdC9, AdC9+MVA, or MVA and sera was obtained 8 weeks later. Antibody titers are expressed in light units and were quantified by a luciferase immunoprecipitation system (LIPS) assay (see Materials and Methods section). (**i**) Immunization with a vector mixture (Ad+MVA, mixed, same syringe and injection site) or a coadministration of two viral vectors (Ad+MVA Coadmin, vectors administered in separate sites) and effect on $CD8^+$ IFN-γ responses. Significant differences were calculated by an ANOVA and *indicate a significant difference between prime and boos. Statistical analyses were using Prism5 software. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, respectively (GraphPad, La Jolla, CA).

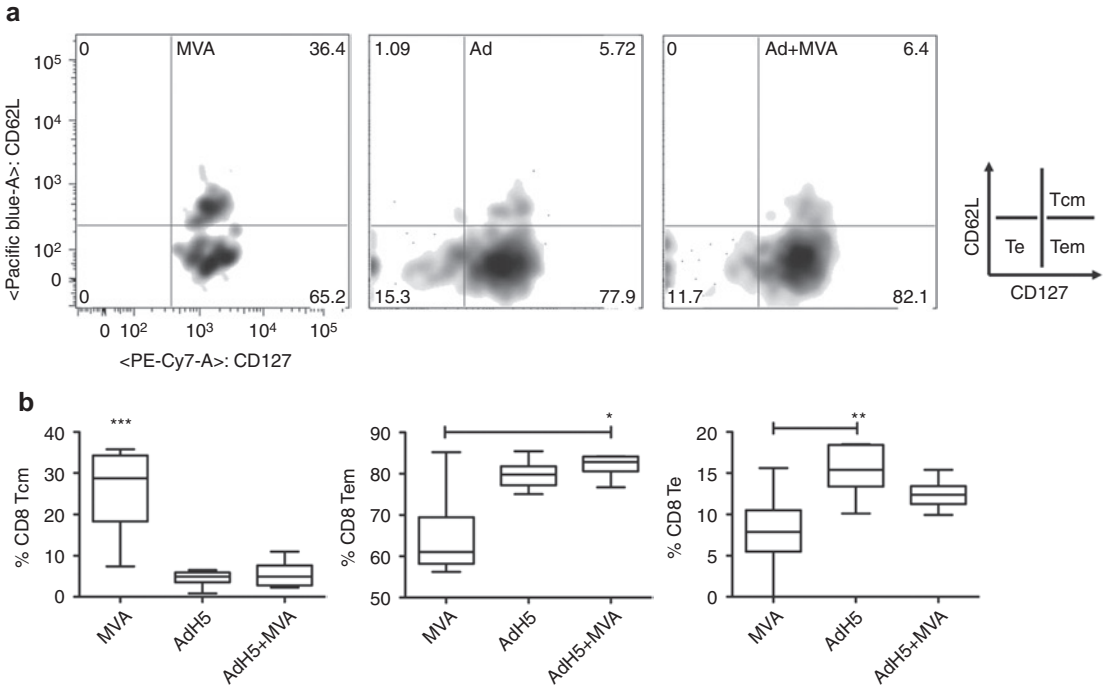


Figure 6 CD8$^+$ memory responses induced by an Ad+MVA vector mixture immunization. Pb9-specific CD8$^+$ T-cell memory responses in BALB/c mice by AdH5 (5 × 10^9 vp/mouse), modified vaccinia virus Ankara (MVA) (1 × 10^6 pfu/mouse) or a vector mixture containing AdH5+MVA-expressing ME.TRAP (5 × 10^9 vp/mouse and 1 × 10^6 pfu/mouse, respectively) were assessed in blood samples taken 8 weeks after immunization. Peripheral blood mononuclear cell (PBMC) were stained using an anti-CD8 antibody, Pb9$^+$ Tetramer (NIH) and anti-CD62L and anti-CD127. (**a**) Density plots were generated after gating the CD8$^+$ and Pb9$^+$ tetramer cells upon immunization with MVA (left), AdH5 (middle), or a mixture using AdH5+MVA (right). Left panel shows a representative mouse of each group and right panel shows the mean and SEM of six mice for each of the CD8$^+$ memory subpopulations. (**b**) Graphs from the same experiment showing the mean and SEM of the CD8$^+$ effector and memory populations induced by the vaccine regimes. Graphs show results of one experiment with $n = 6$ mice per group.

downregulated genes in response to all three vaccines. The number of significantly differentially expressed genes remained high at 24 hours postvaccination but had declined by 72 hours (**Figure 8b**). The highest 30 upregulated genes ($P < 0.01$) at 9 hours and 24 hours postvaccination were found to be identical between Ad, MVA and Ad+MVA mixture, with the majority being type I and II IFNs, IFN-regulated genes and chemokine ligands. At 72 hours postvaccination, the most upregulated genes were still shared by the three vaccines, although most of the viral infection response elements were substituted by genes involved in DNA replication, cell proliferation and differentiation, and protein translation.

A heat-map representation of all genes up- (red) and downregulated (green) in the lymph nodes by at least threefold and reaching the adjusted P value of 0.01 also illustrates the similarities of the gene expression patterns induced by the Ad+MVA mixed vaccine to one or both of the Ad and MVA vaccines. It can be noted however, that the Ad+MVA mixture at 9 hours postvaccination results in a lower level of gene downregulation overall, compared to Ad and MVA alone. In addition, at 72 hours postimmunization we found a threefold up regulation of molecules involved in host defense, in particular CCL3, CCL12, CXCL1, CCL4, CXCL9, CXCL10 (**Figure 8c,d**). These results suggest that the coadministration of Ad and MVA modified their rate of immune activation, although no specific pathway was identified in the analyses performed.

DISCUSSION

Viral-vectored vaccines represent one of the most promising vaccination approaches for some infectious diseases where a preventative vaccine still remains to be developed. Promising results have been obtained in clinical trials using MVA as a tuberculosis vaccine,[19] and efficacy has been demonstrated in trials for HIV using a canarypox vector as part of a prime-boost regime;[20] in malaria trials using fowlpox 9, MVA[21] and chimpanzee Ad as a pre-erythrocytic malaria vaccine (K.J. Ewer, G.A. O'Hara, C.J.A. Duncan, K.A. Collins, S.H. Sheehy, A. Reyes-Sandoval *et al.*, manuscript submitted). The use of viral vectors as vaccines has required heterologous prime-boost strategies to maximize immunogenicity and efficacy.[21] In an attempt to enhance their efficacy while potentially simplifying the logistics of vaccine delivery, we assessed the effect on the immune response of the administration of mixed Ad and MVA, two of the most clinically advanced viral vectors, delivered as part of the same formulation.

We explored the effects of the vector mixture on the adaptive immune responses. We chose a mouse malaria model where a pre-erythrocytic vaccine has shown the induction of protective CD8$^+$ T-cell responses and for which we have a challenge model.[8,13] Our results indicated that intermediate doses for both vectors (5 × 10^9 vp for Ad and 1 × 10^6 pfu for MVA) elicited optimal immunogenicity and protection. We initially reasoned that higher doses of the vector mixture (1 × 10^{10} vp of Ad and 1 × 10^7 pfu of MVA) could enhance efficacy but a detrimental effect was observed for

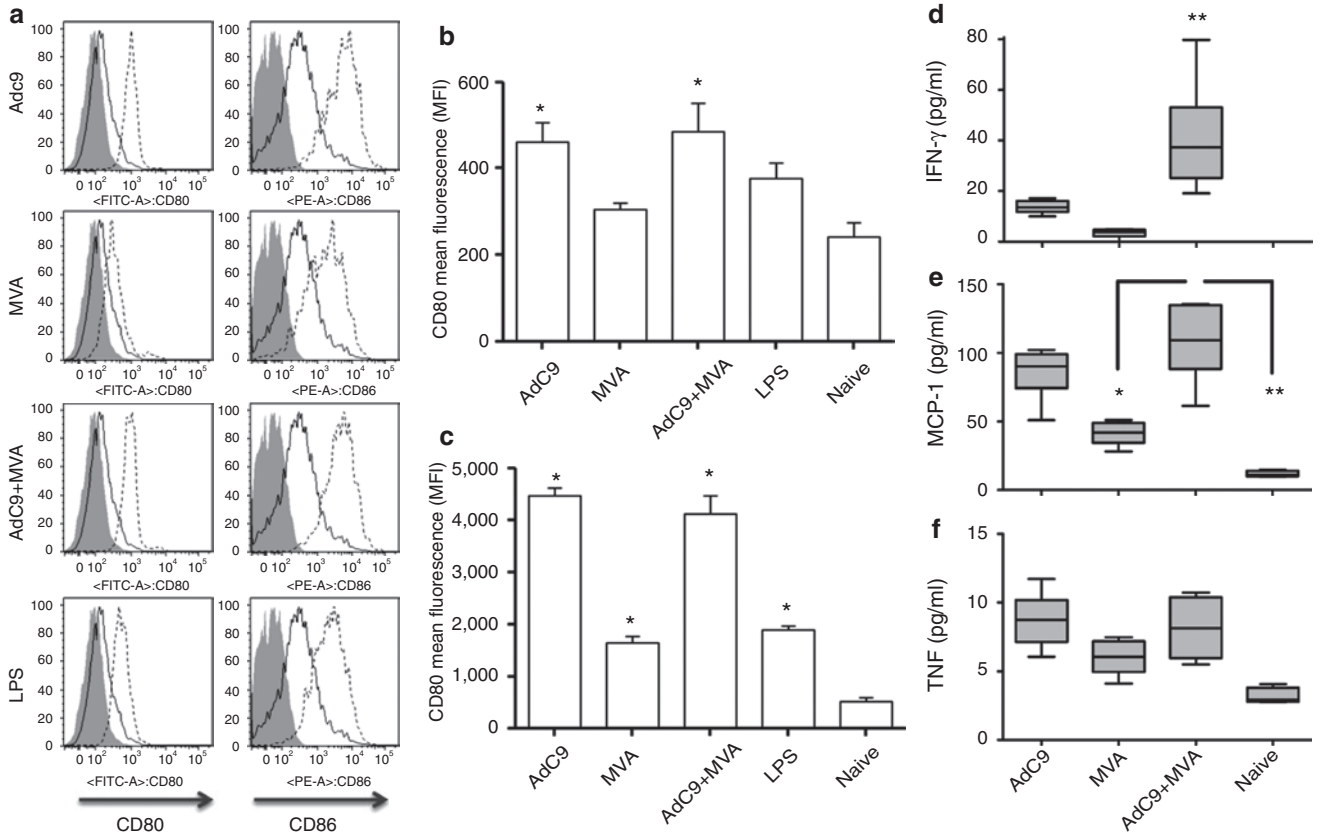


Figure 7 ***In vivo*** **activation of the innate immune system by an Ad+MVA immunization.** (**a**) Activation of dendritic cells by adenovirus (Ad), modified vaccinia virus Ankara (MVA), and Ad+MVA vaccines. Groups of BALB/c mice (n = 10) were immunized intradermally in the ear pinna with AdC9 ME.TRAP (5×10^9 vp/mouse), MVA ME.TRAP [1×10^6 plaque-forming unit (pfu)/mouse], AdC9+MVA ME.TRAP vector mixture (5×10^9 vp and 1×10^6 pfu, respectively) and 10 µg lipopolysaccharide (LPS). Twenty-four hours later, draining auricular lymph nodes were excised and cells were stained with anti-mouse CD11c to quantify the percentage of live $CD11c^+$ cells upregulating $CD80^+$ and $CD86^+$. Representative histogram overlay plots are presented in (**a**) where solid and dotted lines are expression levels of naive and immunized mice, respectively. Gray filled histograms represent unstained background expression level. Graphs displaying the mean fluorescence intensity for CD80 (**b**) and CD86 (**c**). Significant differences of $P < 0.05$ compared to naive were calculated using one way ANOVA analysis. (**d–f**) Quantitative detection of multiple cytokines in mouse serum. Serum cytokines were quantified after immunization using a BD CBA mouse inflammation kit. Figure panels show relevant results where differences between regimes were detected for (**d**) interferon (IFN)-γ; (**e**) MCP-1; and (**f**) tumor necrosis factor (TNF). Data show mean and SEM. Statistical analysis was performed using an ANOVA. Data was obtained from two independent experiments.

both immunogenicity and protection. This result is in agreement with a recent report from Yashima *et al.* where high doses of Ad (10^{10} vp) and MVA (10^7 pfu) were coadministered and a suppression of the antigen-specific $CD8^+$ T cells was observed.[22]

One of the major hurdles to overcome in viral vector vaccination is the dampening effect of the antivector immunity on the antigen-specific T-cell responses. Natural infections with Ad induce neutralizing antibodies that limit the virus transduction, protein expression and therefore decrease the transgene-specific T-cell responses.[23] Moreover, priming with a recombinant adenoviral vector prevents further expansion of T-cell responses by a subsequent homologous boost, reducing at the same time the breadth of the T-cell responses and limiting their ability to control an infection.[24] This has prompted the scientific community to develop novel immunization regimes using alternative adenoviral serotypes that do not circulate in human populations.[8,24]

Additionally, multiple approaches have been developed to circumvent the antivector responses elicited by natural exposure or vaccination. These include relatively simple tools such as the use of heterologous prime-boost to efficiently amplify the immune responses.[25,26] and more sophisticated approaches that combine modern immunology and structural biology techniques to re-engineer an adenoviral vector with modified hypervariable regions of the hexon thus helping the chimeric virus evade the antivector immunity.[27] The spectrum of developments also includes the chemical modification of viral surface proteins to mask the neutralizing epitopes[28] and the use of vectors engineered from rare or alternative serotypes.[13,24] Most, if not all of these approaches aim at modifying one particular vector at a time, while the vector mixture described here would facilitate the use of one vector multiple times as well as the application as a heterologous mixed prime-boost regime, which in the case of the AdC9+MVA-prime followed by an AdC7+MVA-boost was found to be the most protective vaccination regime that nevertheless presents increased difficulties in production and delivery that may counteract the modestly superior performance compared to the homologous mixed vaccine.

To understand the underlying mechanism(s), we have investigated the effect of the mixture on innate immune responses and on

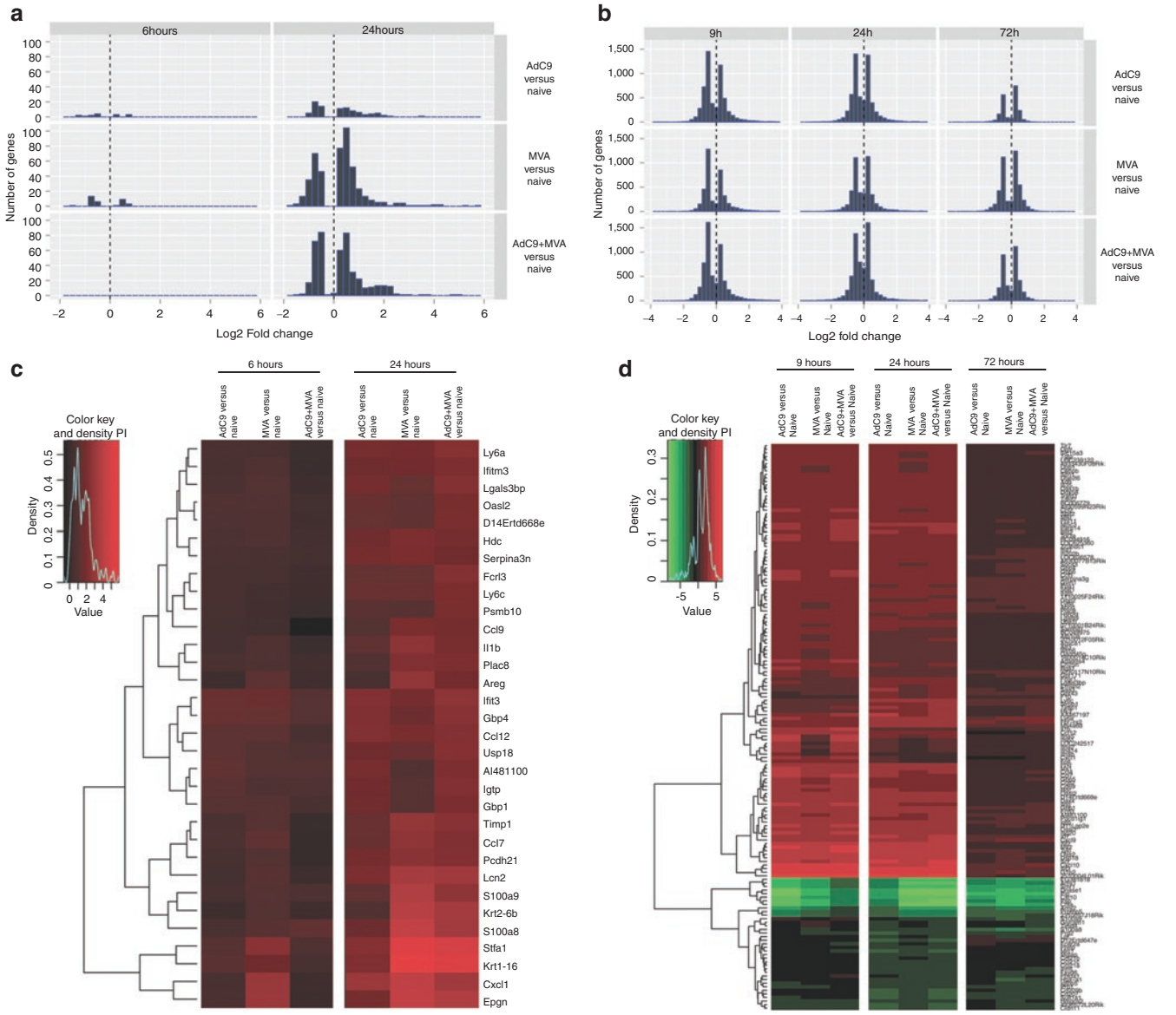


Figure 8 Microarray analysis in ear and lymph node samples after immunization with an AdC9+MVA vector mixture. Total number and fold change of significantly ($P < 0.05$) up- and downregulated genes in the (**a**) ear biopsies and (**b**) lymph nodes following vaccination with AdC9, modified vaccinia virus Ankara (MVA) or AdC9+MVA mixture, determined as differential gene expression relative to nonimmunized samples. The analyzed time-points for each set of samples are indicated. (**c**) Heat map of significantly ($P < 0.01$) differentially expressed genes in the (**c**) ear biopsies and (**d**) lymph node following AdC9, MVA, or mixed AdC9+MVA vaccination. Only genes reaching threefold change in expression are included. Data is available at the public functional genomics data repository Gene Expression Omnibus (GEO), GEO accession number GSE30564

the maturation status of DCs. Previous reports have shown both stimulation and maturation of DCs by both chimpanzee (AdC68) and human Ad (AdH5) *in vitro* and *in vivo*.[29] Effects of MVA on DCs are controversial, with some reports describing a stimulatory effect[18,30] while others observing an inhibitory effect.[31–33] In this report, we show that MVA was able to marginally upregulate the costimulatory molecules of DCs, while AdC9 appeared to be a stronger DC stimulant *in vivo*. Furthermore, the addition of MVA to AdC9 did not dampen the stimulatory properties of the latter; indeed, there were differences in early responses systemically, as evidenced by significantly higher levels of IFN-γ in Ad and MVA (Ad+MVA)-treated animals relative to single agent treated animals.

We attempted to further understand specific pathway(s) induced by the mixture using transcriptional studies on tissues from the immunization site, or the lymph nodes draining it. One finding was of a threefold upregulation of a group of chemotactic molecules (CCL3, CCL12, CXCL1, CCL4, CXCL9, CXCL10) compared to single vectors. This group of molecules, in particular CCL3 and CCL4, have been shown to enhance immunity by attracting $CD8^+$ T cells to the sites where antigen-presenting and $CD4^+$ T cells interact in lymph nodes.[34] Moreover, blocking CCL3 and CCL4 activity reduces the recruitment of naive $CD8^+$ T cells in lymph nodes and decreases the ability to promote $CD8^+$ T cell generation.[34] Of interest, chemokines such as CCL4 (MIP 1-β) have been using as genetic adjuvants and have shown to enhance

transgene-specific immune responses.[35] Thus, enhanced cellular recruitment postvaccination may contribute to the enhanced cellular immune response observed.

In conclusion, Ad and MVA viral vectors can be administered as a mixture to enhance protection against pre-erythrocytic malaria in a model that relies on the induction of antigen-specific $CD8^+$ responses to confer protection. The Ad+MVA mixed vector immunization acts as formulation that provides an "internal" prime-boost to enhance the $CD8^+$ responses directed toward the shared transgenic antigens at the expense of the nonshared antigens from both vectors to significantly decrease the antivector immunity. This effect permits repeated readministration of the vector mixture as a homologous prime-boost regimen, reducing the potential problem of antivector immunity and offering simplified deployment as a single product rather than as heterologous prime-boost regimens.

MATERIALS AND METHODS

Mice and immunizations. Female BALB/c mice 6 weeks of age were purchased from Harlan, Shardlow, UK. Viral vectors were administered intradermally into ear pinnae or intramuscularly in the gastrocnemius muscle by delivering a final volume of 25 µl per limb or ear (50 µl total volume per mouse). The concentration of viral vectors in endotoxin-free phosphate-buffered saline (Sigma, Dorset, UK) was 1×10^6 pfu/50 µl for MVA and 5×10^9 vp/50 µl for adenoviral (Ad) vectors, unless where specified otherwise (**Figure 1**). For all other experiments, Ad+MVA vector mixtures consisted of a single preparation containing both vectors at the same concentrations as the vector control (1×10^6 pfu for MVA plus 5×10^9 vp for Ad in 50 µl of phosphate-buffered saline).

Ethics statement. All animals and procedures were used in strict accordance with the terms of the UK Home Office Animals Act law. Procedures were approved by the University of Oxford Animal Care and Ethical Review Committee (PPL 30/2414). Immunizations were performed under anesthesia and all efforts were made to minimize suffering and reduce animal numbers.

Viral vectors. Vectors expressing the transgene ME.TRAP,[12,36] Pb9, and GFP[37] have been described previously. The insert ME.TRAP is a hybrid transgene of 2,398 bp encoding a protein of 789 amino acids. The ME string contains the BALB/c H-$2K^d$ epitope Pb9 (CS252–260, SYIPSAEKI) from the *P. berghei* malaria CS protein among a number of other human B- and T-cell epitopes.[38] AdC7, AdC9, ChAd63, AdH5, and MVA vectors expressing ME.TRAP were constructed and propagated as described previously.[6,12,39] Design and construction of Ad and MVA vectors expressing the *M. tuberculosis* Ag 85A has been described earlier.[40–42] AdHu5 expressed the E3 protein after cloning the *E3L* open-reading frame of MVA under control of the human cytomegalovirus immediate early promoter was derived and prepared as previously described.[13,42]

Ex vivo *IFNγ ELISPOT.* Isolated splenocytes or PBMCs were treated with ACK to remove red blood cells and then cultured for 18–20 hours on IPVH-membrane plates (Millipore, Watford, UK) with the immunodominant H-$2K^d$-restricted epitope Pb9 (SYIPSAEKI) at a final concentration of 1 µg/ml. ELISPOT was performed as previously described.[8,13] To analyze the subdominant responses to TRAP, PBMCs were stimulated with seven 20mer peptides covering subdominant TRAP epitopes identified in BALB/c mice (IRLHSDASKNKEKALIIIRS, KEKALIIIRSLLSTNLPYGR, TDGIPDSIQ DSLKESRKLSD, GQGINVAFNRFLVGCHPSDG, KCNLYADSAWENVK NVIGPF, TASCGVWDEWSPCSVTCGKG and EPLDVPDEPEDDQPR PRGDN). Anti-Ad $CD8^+$ immune responses were assessed by stimulating PBMCs with the Ad hexon-specific H-$2K^d$-restricted epitope $hex_{486–494}$ (KYSPSNVKIA) or the Ad DNA-binding protein-specific L^d-restricted epitope $dbp_{413–421}$ (LPKLTPFALA) previously described.[15]

The $CD8^+$ T-cell response to MVA was measured using stimulation with synthetic peptides corresponding to the two immunodominant determinants originally identified in BALB/c mice, E3 and F2(G).[16] In addition, we modified the more recently discovered SI9 peptide[43] to make its sequence identical to that present within the MVA insert (Gly to Ser substitution at position 1), here referred to as C6(S), analogous to the derivation of F2(G) from vaccinia virus F2 peptide.[16]

Neutralizing antibodies against Ad. BALB/c mice were immunized with AdH5 or AdH5+MVA mixture and 2 weeks later the neutralizing antibodies against AdH5 were measured by incubating heat-inactivated mouse sera with AdH5 expressing SEAP (secreted alkaline phosphatase) for 60 minutes at 37 °C in 5% CO_2. The virus was then added to HEK 293 cells at various multiplicity of infection (4,000–0.98 by performing serial dilutions) and the reporter gene was measured using the Phospha-Light SEAP Reporter Gene Assay System (Applied Biosystems, Paisley, UK) using the Plate reading luminometer Wallac MicroBeta TriLux (Perkin Elmer, Cambridge, UK). The neutralizing antibody titre was defined as the dilution of test serum at which reporter gene expression (secreted alkaline phosphatase) in permissive cells fell to 50% of the expression in cells infected in the absence of immune serum.

Antibody responses against TRAP. BALB/c mice were immunized with AdC9, AdC9+MVA mixture or MVA-expressing ME.TRAP ($n = 6$). Serum was obtained 8 weeks later and antibodies against TRAP were quantified by a luciferase immunoprecipitation system assay.[44,45] Briefly, TRAP antigen was expressed in cell culture as recombinant *Renilla* luciferase (Ruc)-antigen fusion. The luciferase immunoprecipitation system assay was initiated by incubating Ruc-antigen extract with mouse sera in microtiter wells. The antibody-antigen mixture is then transferred to a 96-well filter plate containing protein A/G beads to capture immunoglobulin G molecules. After washing the filter plate containing the protein A/G beads, antibody bound Ruc-antigen is measured by the addition of coelenterazine substrate and light units are measured with a luminometer.

Activation of DCs. Groups of BALB/c mice ($n = 4$) were immunized intradermally with AdC9 ME.TRAP (5×10^9 vp/mouse), MVA ME.TRAP (1×10^6 pfu/mouse) or an AdC9+MVA ME.TRAP vector mixture (5×10^9 vp and 1×10^6 pfu, respectively). Twenty-four hours later, draining superficial cervical lymph nodes were excised and processed to obtain a single-cell suspension in Dulbecco's modified Eagle's medium media supplemented with 4 mmol/l glutamine, 10% fetal calf serum, and 100 U/ml penicillin/streptomycin. Cells were stained in phosphate-buffered saline for 30 minutes at 4 °C using the LIVE/DEAD Fixable Dead Cell Staining Kit (violet fluorescent reactive dye; Invitrogen, Paisley, UK). Fcγ II receptors were blocked with anti-CD16/CD32 FcγIII/II Receptor (2.4G2; BD/Pharmingen, Oxford, UK) for 30 minutes at 4 °C. DC markers were stained using anti-mouse CD11c PE-Cy7 (clone N418), CD80 FITC (clone 16-10A1), and CD86 PE (clone GL1) (eBioscience, Hatfield, UK) for 30 minutes at 4 °C. Samples were acquired on an LSR II cytometer (BD, Oxford, UK) and analyzed using FlowJo software (Tree Star, Ashland, OR).

Cell staining and flow cytometry. For intracellular cytokine staining, ACK buffer-treated splenocytes were incubated for 5 hours in the presence of 1 µg/ml Pb9, 1 µl/ml Golgi-Plug, and 1 µl/ml Golgi-Stop (BD) and anti-CD107a PE (clone 1D4B) (eBioscience) at a final dilution of 1:200. Phenotypic analysis of $CD8^+$ T cells was performed by intracellular cytokine staining using previously described antibody clones,[13] specifically anti-CD8 PerCP-Cy5.5 (clone 53-6.7) (BD), anti-IFN-γ APC (clone XMG1.2), anti-IL-2 PE-Cy7 (clone JES6-5H4), anti-TNF-α FITC (Clone MP6-XT22), all from eBioscience). Nonspecific binding of antibodies was prevented by incubating with anti-CD16/CD32 Fcγ III/II Receptor (2.4G2; BD/Pharmingen) before staining. The Pb9 tetramer was produced by the NIH

tetramer facility (MHC tetramer core facility, Emory University Vaccine Center, Atlanta, GA) using the peptide SYIPSAEKI (Proimmune, Oxford, UK). Flow cytometric analyses were performed using an LSRII (BD Biosciences). Data were analyzed with either FACSDiva (BD) or FlowJo (Tree Star) software. Analysis of multifunctional $CD8^+$ T-cell responses was performed using a Boolean analysis generated in FlowJo to be used in, Pestle and SPICE 4.0 software, kindly provided by M. Roederer (NIH, Bethesda, MD).[46]

Quantitative detection of multiple cytokines in mouse serum. Serum cytokines were quantified after immunizing BALB/c mice ($n = 6$) intradermally AdC9 ME.TRAP (5×10^9 vp/mouse), MVA ME.TRAP (1×10^6 pfu/mouse) or an AdC9+MVA ME.TRAP vector mixture (5×10^9 vp and 1×10^6 pfu, respectively). Twenty-four hours later, sera was obtained and cytokines were quantified using a BD Cytometric Bead Array Mouse Inflammation Kit (BD Biosciences) following manufacturer's recommendations. Statistical analysis was performed using an ANOVA (Prism; Graphpad, La Jolla, CA).

Parasite challenge. *P. berghei* (ANKA strain clone 234) sporozoites were isolated from salivary glands of female *Anopheles stephensi* mosquitoes. Parasites were resuspended in RPMI-1640 media and each mouse received a total of 1,000 sporozoites intravenously. Blood samples were taken daily from day 5 to day 20; blood smears were stained with Giemsa and observed under a light microscope for the presence of parasites within the red blood cells.

The transgenic *P. berghei* parasites (PbGFP-Luc_{con}) used in the study expressed fusion GFP (mutant 3) and firefly luciferase (*LucIAV*) genes under the control of constitutive EF1a promoter.[47] The parasites were generated by a stable double-crossover homologous integration of the transgene into P230p locus in the reference line of *P. berghei* ANKA cl15cy1. The transgenic parasites were kindly provided by Dr Oliver Billker from Wellcome Trust Sanger Institute, Hinxton, UK.

Statistical analysis of the survival to a sporozoite challenge. Statistical differences between groups after challenge were assessed by Kaplan–Meier analysis to compare the survival curves using the log rank test in prism (Graphpad). Additionally, we assessed how rapidly the mice became infected by using the Hazard ratio, which is based on the slope of the survival curve for each group (Prism, Graphpad).

In vivo *imaging after malaria sporozoite challenge.* Bioluminescent luciferase signal was detected by imaging the whole animals using the *in vivo* IVIS 200 imaging system (Caliper Life Sciences, Runcorn, UK) as described before.[48] Briefly, 44 hours after the intravenous injection with 1,000 transgenic *P. berghei* sporozoites the mice were anaesthetized in batches of three using isofluorane, their fronts shaved and D-luciferin (Synchem Laborgemeinschaft OHG, Felsberg, Germany) injected into the neck at a concentration of 100 mg/kg in sterile phosphate-buffered saline (Sigma). Animals were imaged for 120 seconds at binning value of 8 and FVO of 12.8 cm, 8 minutes after the injection of D-luciferin. Mice were kept anaesthetized throughout the whole procedure. Quantification of bioluminescence signal was performed using Living Image 4.2 software (Caliper Life Sciences). The region of interest were set around the liver area of the mouse body and kept constant for all of the animals. The measurements were expressed as a total flux of photons per second of imaging time.

Microarray analysis of gene expression following immunization with viral-vectored vaccines. Six-week-old female BALB/c mice ($n = 4$ per group) were immunized with either AdC9-ME.TRAP (5×10^9 vp/mouse), MVA-ME.TRAP (10^6 pfu/mouse) or a combination of the two. The vaccines were administered intradermally, half dose into each ear. Ear biopsies of the immunization site and superficial cervical lymph nodes were collected into RNAlater (Ambion, Applied Biosystems) at specified time points (6 hours and 24 hours for ear biopsies and 9, 24, and 72 hours for lymph nodes) along with samples from nonimmunized animals. Within 2 hours of sample collection total RNA was extracted using the miRNeasy kit (Qiagen, Crawley, UK).

Subsequent sample processing and hybridization was carried out by Source Bioscience, Nottingham, UK. Briefly, the quantity and quality of the extracted RNA was evaluated and the best quality samples used for hybridization to whole mouse genome microarray chips (MouseRef8 v2.0; Illumina, Essex, UK) using three or four replicates for each time-point/treatment. The "quantile" normalization method was used for all reported analyses and background correction implemented within Illumina's Beadstudio software. Normalization corrects for the differences in expression levels across a chip and between chips and is performed so that the arrays are comparable.

Quality assessment of the expression data (*e.g.*, probe intensity, variation among replicates) was performed using the Bioconductor package, employing "limma" and "Beadarray" libraries. Differential expression data between vaccinated and nonimmunized samples contained various statistics including $\log_{(2)}$-fold change and *P* value (adjusted for multiple testing using Benjamini and Hochberg's correction). Differential expression results were further examined using the Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics resource (National Institute of Allergy and Infectious Diseases).[49] Microarray data was submitted to the public functional genomics data repository Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) and a GEO accession number was assigned (GSE30564).

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REFERENCES

1. Rollier, CS, Reyes-Sandoval, A, Cottingham, MG, Ewer, K and Hill, AV (2011). Viral vectors as vaccine platforms: deployment in sight. *Curr Opin Immunol* **23**: 377–382.
2. McCoy, K, Tatsis, N, Korioth-Schmitz, B, Lasaro, MO, Hensley, SE, Lin, SW *et al.* (2007). Effect of preexisting immunity to adenovirus human serotype 5 antigens on the immune responses of nonhuman primates to vaccine regimens based on human- or chimpanzee-derived adenovirus vectors. *J Virol* **81**: 6594–6604.
3. Belnoue, E, Costa, FT, Frankenberg, T, Vigário, AM, Voza, T, Leroy, N *et al.* (2004). Protective T cell immunity against malaria liver stage after vaccination with live sporozoites under chloroquine treatment. *J Immunol* **172**: 2487–2495.
4. Anderson, RJ, Hannan, CM, Gilbert, SC, Laidlaw, SM, Sheu, EG, Korten, S *et al.* (2004). Enhanced CD8+ T cell immune responses and protection elicited against *Plasmodium berghei* malaria by prime boost immunization regimens using a novel attenuated fowlpox virus. *J Immunol* **172**: 3094–3100.
5. Capone, S, Reyes-Sandoval, A, Naddeo, M, Siani, L, Ammendola, V, Rollier, CS *et al.* (2010). 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.
6. Gilbert, SC, Schneider, J, Hannan, CM, Hu, JT, Plebanski, M, Sinden, R *et al.* (2002). Enhanced CD8 T cell immunogenicity and protective efficacy in a mouse malaria model using a recombinant adenoviral vaccine in heterologous prime-boost immunisation regimes. *Vaccine* **20**: 1039–1045.
7. Reyes-Sandoval, A, Harty, JT and Todryk, SM (2007). Viral vector vaccines make memory T cells against malaria. *Immunology* **121**: 158–165.
8. Reyes-Sandoval, A, Berthoud, T, Alder, N, Siani, L, Gilbert, SC, Nicosia, A *et al.* (2010). 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.
9. Hill, AV, Reyes-Sandoval, A, O'Hara, G, Ewer, K, Lawrie, A, Goodman, A *et al.* (2010). Prime-boost vectored malaria vaccines: progress and prospects. *Hum Vaccin* **6**: 78–83.
10. Hutchings, CL, Gilbert, SC, Hill, AV and Moore, AC (2005). Novel protein and poxvirus-based vaccine combinations for simultaneous induction of humoral and cell-mediated immunity. *J Immunol* **175**: 599–606.

11. Hutchings, CL, Birkett, AJ, Moore, AC and Hill, AV (2007). Combination of protein and viral vaccines induces potent cellular and humoral immune responses and enhanced protection from murine malaria challenge. *Infect Immun* **75**: 5819–5826.
12. McConkey, SJ, Reece, WH, Moorthy, VS, Webster, D, Dunachie, S, Butcher, G *et al.* (2003). Enhanced T-cell immunogenicity of plasmid DNA vaccines boosted by recombinant modified vaccinia virus Ankara in humans. *Nat Med* **9**: 729–735.
13. Reyes-Sandoval, A, Sridhar, S, Berthoud, T, Moore, AC, Harty, JT, Gilbert, SC *et al.* (2008). Single-dose immunogenicity and protective efficacy of simian adenoviral vectors against *Plasmodium berghei*. *Eur J Immunol* **38**: 732–741.
14. Reyes-Sandoval, A, Wyllie, DH, Bauza, K, Milicic, A, Forbes, EK, Rollier, CS *et al.* (2011). CD8+ T effector memory cells protect against liver-stage malaria. *J Immunol* **187**: 1347–1357.
15. Schirmbeck, R, Reimann, J, Kochanek, S and Kreppel, F (2008). The immunogenicity of adenovirus vectors limits the multispecificity of CD8 T-cell responses to vector-encoded transgenic antigens. *Mol Ther* **16**: 1609–1616.
16. Tscharke, DC, Woo, WP, Sakala, IG, Sidney, J, Sette, A, Moss, DJ *et al.* (2006). Poxvirus CD8+ T-cell determinants and cross-reactivity in BALB/c mice. *J Virol* **80**: 6318–6323.
17. Bachmann, MF, Wolint, P, Schwarz, K, Jäger, P and Oxenius, A (2005). Functional properties and lineage relationship of CD8+ T cell subsets identified by expression of IL-7 receptor alpha and CD62L. *J Immunol* **175**: 4686–4696.
18. Drillien, R, Spehner, D and Hanau, D (2004). Modified vaccinia virus Ankara induces moderate activation of human dendritic cells. *J Gen Virol* **85**(Pt 8): 2167–2175.
19. Brookes, RH, Hill, PC, Owiafe, PK, Ibanga, HB, Jeffries, DJ, Donkor, SA *et al.* (2008). Safety and immunogenicity of the candidate tuberculosis vaccine MVA85A in West Africa. *PLoS ONE* **3**: e2921.
20. Rerks-Ngarm, S, Pitisuttithum, P, Nitayaphan, S, Kaewkungwal, J, Chiu, J, Paris, R *et al.*; MOPH-TAVEG Investigators. (2009). Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. *N Engl J Med* **361**: 2209–2220.
21. Webster, DP, Dunachie, S, Vuola, JM, Berthoud, T, Keating, S, Laidlaw, SM *et al.* (2005). Enhanced T cell-mediated protection against malaria in human challenges by using the recombinant poxviruses FP9 and modified vaccinia virus Ankara. *Proc Natl Acad Sci USA* **102**: 4836–4841.
22. Yashima, S, Yoshizaki, S, Shinoda, K, Yoshida, A, Kondo, A, Mizuguchi, H *et al.* (2010). Co-administration of viral vector-based vaccines suppresses antigen-specific effector CD8 T cells. *Vaccine* **28**: 3257–3264.
23. Lasaro, MO and Ertl, HC (2009). New insights on adenovirus as vaccine vectors. *Mol Ther* **17**: 1333–1339.
24. Liu, J, O'Brien, KL, Lynch, DM, Simmons, NL, La Porte, A, Riggs, AM *et al.* (2009). Immune control of an SIV challenge by a T-cell-based vaccine in rhesus monkeys. *Nature* **457**: 87–91.
25. Gilbert, SC, Moorthy, VS, Andrews, L, Pathan, AA, McConkey, SJ, Vuola, JM *et al.* (2006). Synergistic DNA-MVA prime-boost vaccination regimes for malaria and tuberculosis. *Vaccine* **24**: 4554–4561.
26. Schneider, J, Gilbert, SC, Hannan, CM, Dégano, P, Prieur, E, Sheu, EG *et al.* (1999). Induction of CD8+ T cells using heterologous prime-boost immunisation strategies. *Immunol Rev* **170**: 29–38.
27. Roberts, DM, Nanda, A, Havenga, MJ, Abbink, P, Lynch, DM, Ewald, BA *et al.* (2006). Hexon-chimaeric adenovirus serotype 5 vectors circumvent pre-existing anti-vector immunity. *Nature* **441**: 239–243.
28. Mok, H, Palmer, DJ, Ng, P and Barry, MA (2005). Evaluation of polyethylene glycol modification of first-generation and helper-dependent adenoviral vectors to reduce innate immune responses. *Mol Ther* **11**: 66–79.
29. Hensley, SE, Giles-Davis, W, McCoy, KC, Weninger, W and Ertl, HC (2005). Dendritic cell maturation, but not CD8+ T cell induction, is dependent on type I IFN signaling during vaccination with adenovirus vectors. *J Immunol* **175**: 6032–6041.
30. Nörder, M, Becker, PD, Drexler, I, Link, C, Erfle, V and Guzmán, CA (2010). Modified vaccinia virus Ankara exerts potent immune modulatory activities in a murine model. *PLoS ONE* **5**: e11400.
31. Kastenmuller, W, Drexler, I, Ludwig, H, Erfle, V, Peschel, C, Bernhard, H *et al.* (2006). Infection of human dendritic cells with recombinant vaccinia virus MVA reveals general persistence of viral early transcription but distinct maturation-dependent cytopathogenicity. *Virology* **350**: 276–288.
32. Jenne, L, Hauser, C, Arrighi, JF, Saurat, JH and Hügin, AW (2000). Poxvirus as a vector to transduce human dendritic cells for immunotherapy: abortive infection but reduced APC function. *Gene Ther* **7**: 1575–1583.
33. Behboudi, S, Moore, A, Gilbert, SC, Nicoll, CL and Hill, AV (2004). Dendritic cells infected by recombinant modified vaccinia virus Ankara retain immunogenicity *in vivo* despite *in vitro* dysfunction. *Vaccine* **22**: 4326–4331.
34. Castellino, F, Huang, AY, Altan-Bonnet, G, Stoll, S, Scheinecker, C and Germain, RN (2006). Chemokines enhance immunity by guiding naive CD8+ T cells to sites of CD4+ T cell-dendritic cell interaction. *Nature* **440**: 890–895.
35. Pinto, AR, Reyes-Sandoval, A and Ertl, HC (2003). Chemokines and TRANCE as genetic adjuvants for a DNA vaccine to rabies virus. *Cell Immunol* **224**: 106–113.
36. Gilbert, SC, Plebanski, M, Harris, SJ, Allsopp, CE, Thomas, R, Layton, GT *et al.* (1997). A protein particle vaccine containing multiple malaria epitopes. *Nat Biotechnol* **15**: 1280–1284.
37. Alcock, R, Cottingham, MG, Rollier, CS, Furze, J, De Costa, SD, Hanlon, M *et al.* (2010). Long-term thermostabilization of live poxviral and adenoviral vaccine vectors at supraphysiological temperatures in carbohydrate glass. *Sci Transl Med* **2**: 19ra12.
38. Schneider, J, Gilbert, SC, Blanchard, TJ, Hanke, T, Robson, KJ, Hannan, CM *et al.* (1998). Enhanced immunogenicity for CD8+ T cell induction and complete protective efficacy of malaria DNA vaccination by boosting with modified vaccinia virus Ankara. *Nat Med* **4**: 397–402.
39. Roy, S, Gao, G, Lu, Y, Zhou, X, Lock, M, Calcedo, R *et al.* (2004). Characterization of a family of chimpanzee adenoviruses and development of molecular clones for gene transfer vectors. *Hum Gene Ther* **15**: 519–530.
40. Forbes, EK, Sander, C, Ronan, EO, McShane, H, Hill, AV, Beverley, PC *et al.* (2008). Multifunctional, high-level cytokine-producing Th1 cells in the lung, but not spleen, correlate with protection against *Mycobacterium tuberculosis* aerosol challenge in mice. *J Immunol* **181**: 4955–4964.
41. McShane, H, Pathan, AA, Sander, CR, Keating, SM, Gilbert, SC, Huygen, K *et al.* (2004). Recombinant modified vaccinia virus Ankara expressing antigen 85A boosts BCG-primed and naturally acquired antimycobacterial immunity in humans. *Nat Med* **10**: 1240–1244.
42. Sridhar, S, Reyes-Sandoval, A, Draper, SJ, Moore, AC, Gilbert, SC, Gao, GP *et al.* (2008). Single-dose protection against *Plasmodium berghei* by a simian adenovirus vector using a human cytomegalovirus promoter containing intron A. *J Virol* **82**: 3822–3833.
43. Oseroff, C, Kos, F, Bui, HH, Peters, B, Pasquetto, V, Glenn, J *et al.* (2005). HLA class I-restricted responses to vaccinia recognize a broad array of proteins mainly involved in virulence and viral gene regulation. *Proc Natl Acad Sci USA* **102**: 13980–13985.
44. Burbelo, PD, Ching, KH, Klimavicz, CM and Iadarola, MJ (2009). Antibody profiling by Luciferase Immunoprecipitation Systems (LIPS). *J Vis Exp*.
45. Burbelo, PD, Ching, KH, Bush, ER, Han, BL and Iadarola, MJ (2010). Antibody-profiling technologies for studying humoral responses to infectious agents. *Expert Rev Vaccines* **9**: 567–578.
46. Roederer, M, Nozzi, JL and Nason, MC (2011). SPICE: exploration and analysis of post-cytometric complex multivariate datasets. *Cytometry A* **79**: 167–174.
47. Janse, CJ, Franke-Fayard, B, Mair, GR, Ramesar, J, Thiel, C, Engelmann, S *et al.* (2006). High efficiency transfection of *Plasmodium berghei* facilitates novel selection procedures. *Mol Biochem Parasitol* **145**: 60–70.
48. Ploemen, IH, Prudêncio, M, Douradinha, BG, Ramesar, J, Fonager, J, van Gemert, GJ *et al.* (2009). Visualisation and quantitative analysis of the rodent malaria liver stage by real time imaging. *PLoS ONE* **4**: e7881.
49. Huang, da W, Sherman, BT and Lempicki, RA (2009). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* **4**: 44–57.