BRIEF COMMUNICATIONS

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Effective induction of high-titer antibodies by viral vector vaccines

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Protein-in-adjuvant vaccines have shown limited success against difficult diseases such as blood-stage malaria. Here we show that a recombinant adenovirus–poxvirus prime-boost immunization regime (known to induce strong T cell immunogenicity) can also induce very strong antigen-specific antibody responses, and we identify a simple complement-based adjuvant to further enhance immunogenicity. Antibodies induced against a blood-stage malaria antigen by this viral vector platform are highly effective against *Plasmodium yoelii* parasites in mice and against *Plasmodium falciparum in vitro*.

Subunit vaccine development for diseases susceptible to antibody-mediated immunity has classically focused on recombinant protein-in-adjuvant formulations. These require multiple immunizations to induce antibody responses of a protective magnitude, and clinical trials of such candidate blood-stage malaria vaccines remain disappointing[1]. Recombinant protein vaccines are hampered by the inherent difficulties of reliably purifying correctly folded proteins and of formulating them in effective human-compatible adjuvants. Viral vaccine vectors, deployed in heterologous prime-boost regimes, have been developed to induce T cell responses targeting intracellular pathogens[2]. These vectors, including adenovirus human serotype 5 (AdHu5) and the orthopoxvirus modified vaccinia virus Ankara (MVA), are suitable for human use[2,3] and circumvent many of the difficulties associated with protein vaccines. Here we describe AdHu5 and MVA vaccines that express the blood-stage malaria antigen merozoite surface protein-1 (MSP-1) and report that vectored vaccines can work by inducing very high titer antibodies. We also show that a simple complement-based adjuvant can enhance antibody production and $CD4^+$ and $CD8^+$ T cell responses induced by vectored vaccines. Notably, this approach provides protection against *P. yoelii* in mice and strong antibody-mediated growth inhibitory activity (GIA) in a standardized *in vitro* assay against blood-stage *P. falciparum*.

Studies using an AdHu5 prime and an MVA or replication-competent vaccinia virus boost have been described[4,5], but the dependence of AdHu5-MVA vaccine immunogenicity on prime-boost interval has not been assessed. We prepared AdHu5 and MVA vaccines (Ad42 and M42, respectively) expressing the 42-kDa region of MSP-1 ($MSP\text{-}1_{42}$) from the murine malaria parasite *P. yoelii* (**Supplementary Methods** online). BALB/c mice were immunized with Ad42, and antibody responses specific to the protective C-terminal 19-kDa region ($MSP\text{-}1_{19}$) of the antigen[6,7] were monitored by ELISA over time. Total IgG antibody responses to $MSP\text{-}1_{19}$ were significantly higher at day 56 compared to day 14 (**Fig. 1a**). Mice were then primed with Ad42 and boosted after 14 d (2 weeks) or 56 d (8 weeks) with M42 (prime-boost immunization now referred to as AdM42), and responses to $MSP\text{-}1_{42}$ were examined 14 d after the boost. Significantly higher total IgG responses were induced after the 8-week prime-boost regime (**Fig. 1b**). No intracellular interferon-γ (IFN-γ) production was detected in splenic T cells after re-stimulation with peptides corresponding to $MSP\text{-}1_{19}$ (data not shown), but strong $CD8^+IFN\text{-}\gamma^+$ and relatively weak $CD4^+IFN\text{-}\gamma^+$ T cell responses were measured in response to $MSP\text{-}1_{33}$ (**Fig. 1c**). In agreement with these data, $MSP\text{-}1_{19}$ is known to be refractory to antigen processing[8], and the $CD4^+$ T helper cell response against $MSP\text{-}1_{33}$ is likely to be crucial for antibody responses to $MSP\text{-}1_{19}$.

Immunized mice were subsequently challenged intravenously with 1×10^4 red blood cells infected with parasites (pRBCs) 2 weeks after boost (**Supplementary Table 1a** online). All of the naive unimmunized control mice (**Fig. 1d**) and those that received the AdM42 two-week prime-boost regime (**Fig. 1e**) succumbed to *P. yoelii* infection within 6 d. The AdM42 8-week regime protected 76% mice against challenge ($P = 0.001$ compared to the two-week regime; **Fig. 1f**). Protection against blood-stage malaria is thus achievable with viral-vectored vaccines when using an extended prime-boost interval. AdHu5 vaccines have shown strong immunogenicity in other studies, and the requirement described here for a prolonged prime-boost interval is probably essential for the formation of optimal B cell and T helper cell memory populations, which are more effectively boosted by MVA. Similar findings about the interval were reported with AdHu5 and replication-competent vaccinia virus targeting the *P. yoelii* circumsporozoite protein[4]. Other prime-boost regimes including DNA-MVA, DNA-AdHu5, AdHu5-AdHu5 and MVA-AdHu5 did not induce high-titer antibodies and were completely nonprotective in this model (S.J.D., A.C.M., S.C.G. and A.V.S.H., unpublished data).

In an attempt to further improve vaccine efficacy, we produced AdHu5 and MVA vectors expressing *P. yoelii* $MSP\text{-}1_{42}$ fused at the C terminus to a complement-based 'molecular adjuvant'. Complement C4b-binding protein (C4bp) is a circulating soluble inhibitor of the complement pathways[9]. The C-terminal 54 amino acids of the alpha chain of mouse C4bp (mC4bp) enhance antibody responses when fused to an antigen; recombinant *P. yoelii* $MSP\text{-}1_{19}$ fused to mC4bp was highly immunogenic when administered in saline and induced protective antibodies in mice[10]. A similar approach with complement

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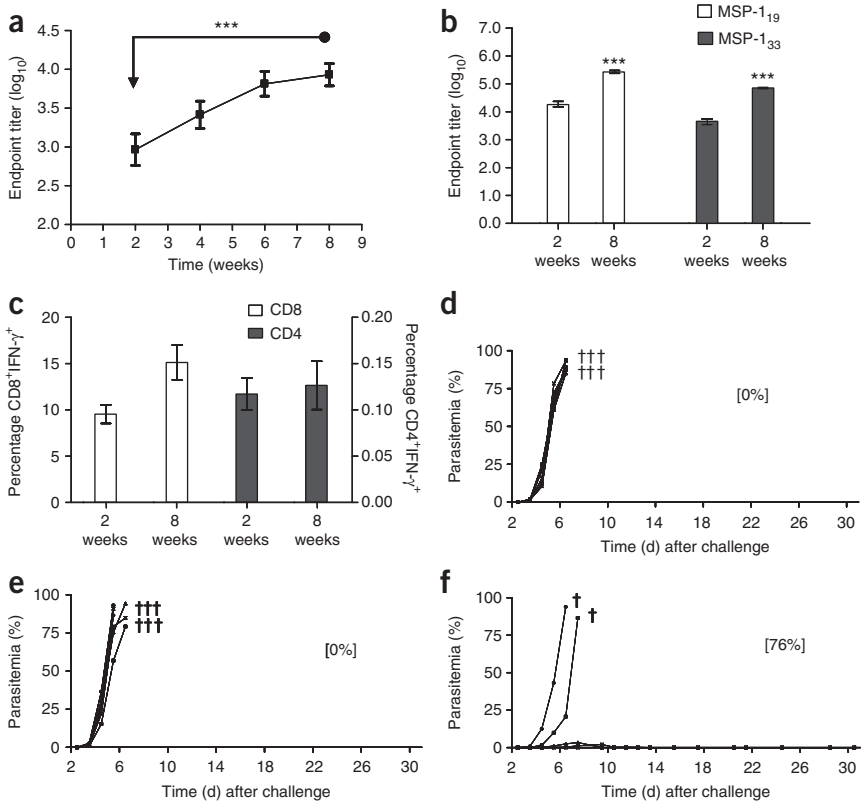

Figure 1 AdM42 vaccine–induced immune responses and protection against blood-stage *P. yoelii* are dependent on the prime-boost interval in BALB/c mice. (**a**) Mice were immunized intradermally with 5×10^{10} Ad42 viral particles, and total IgG serum antibody responses to MSP-1_{19} were measured over time by ELISA. (**b**) Mice were primed as in **a** with Ad42 and boosted intradermally with 5×10^7 plaque-forming units M42 2 or 8 weeks later. Total IgG serum antibody responses to MSP-1_{19} and MSP-1_{33} were measured by ELISA 14 d after boost. (**c**) CD8$^+$ and CD4$^+$ T cell IFN-γ responses after re-stimulation with MSP-1_{33} peptides were assessed in the spleen by intracellular cytokine staining. The mean responses ± s.e.m. are shown ($n \geq 6$ mice per group). *** $P \leq 0.001$ in **a** (paired *t*-test comparing responses between time points) and **b** (independent *t*-test comparing responses between the two groups). Similar results were obtained in two or three independent experiments. (**d**–**f**) Naive unimmunized control mice (**d**), AdM42-immunized mice boosted after 2 weeks (**e**) or AdM42-immunized mice boosted after 8 weeks (**f**) were challenged intravenously with 1×10^4 *P. yoelii* pRBCs on day 14 after boost. Parasitemia was measured as described in the **Supplementary Methods** from day 2 after challenge. Representative results are shown ($n = 6$ mice per group). Percentage survival, including all repeat experiments, is indicated in square parentheses (see **Supplementary Table 1a**). Crosses indicate the point at which the mice were killed.

protein C3d has shown promise when using plasmid DNA[11], but the expression of such complement-based adjuvants by viral vectors has not been described.

We immunized BALB/c mice with either AdM42 or AdHu5-MVA vectors encoding MSP-1_{42} fused to mC4bp (AdM42-C4bp) using an 8-week prime-boost interval and assayed immune responses as before. Mice immunized with vectors expressing MSP-1_{42}–C4bp developed significantly higher antigen-specific total IgG titers (**Fig. 2a**). We focused on the IgG isotypes induced against the protective MSP-1_{19} domain[6,7]. Of note, the use of vectors expressing MSP-1_{42}–C4bp led to a shift toward T helper type 1 (T_H1) associated antigen-specific IgGs, with significantly elevated levels of IgG2a, IgG2b and IgG3 and reduced levels of IgG1 compared to those in mice immunized with vectors expressing MSP-1_{42} (**Fig. 2b**). No differences in antibody avidity[11] were observed between the two groups (data not shown). There was also a significant ($P = 0.02$) increase in the percentage of MSP-1_{33}–specific splenic CD4$^+$ IFN-γ$^+$ T cells in the mice immunized with the MSP-1_{42}–C4bp vectors (**Fig. 2c**), possibly accounting for the

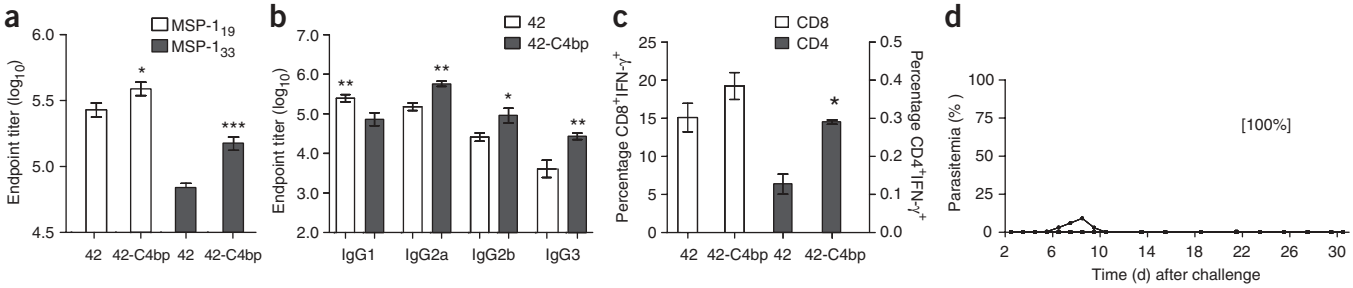

Figure 2 AdM42-C4bp vaccine–induced immune responses and protection against blood-stage *P. yoelii*. BALB/c mice were immunized as in **Figure 1b** with either AdM42 or AdM42-C4bp regimes and an 8-week prime-boost interval. (**a**,**b**) Total IgG serum antibody responses to MSP-1_{19} and MSP-1_{33} (**a**) or IgG isotype responses against MSP-1_{19} (**b**) measured by ELISA 14 d after boost. (**c**) CD8$^+$ and CD4$^+$ T cell IFN-γ responses were assessed in the spleen as in **Figure 1c**. The mean responses ± s.e.m. are shown ($n \geq 6$ mice per group). * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$ in **a**–**c** (independent *t*-test comparing responses between the two groups). Similar results were obtained in at least two independent experiments. (**d**) AdM42-C4bp–immunized mice were challenged as in **Figure 1d**–**f** with 1×10^4 *P. yoelii* pRBCs. A representative result is shown ($n = 6$ mice). Percentage survival, including all repeat experiments, is indicated in square parentheses. Naive unimmunized control mice ($n = 6$) succumbed to infection as in **Figure 1d** (see **Supplementary Table 1a**). All animal experimentation and procedures were carried out under the terms of the UK Animals (Scientific Procedures) Act Home Office Project Licence and were approved by the University of Oxford Animal Care and Ethical Review Committee.

increased amount of T_H1-type IgG in this group. The percentage of $CD8^+$ IFN-γ^+ T cells was also higher but not significantly so (**Fig. 2c**).

After blood-stage malaria challenge, all of the mice immunized with the AdM42-C4bp regime were protected, with $P = 0.03$ compared to the AdM42 regime (**Supplementary Table 1a** and **Fig. 2d**). Ten out of the seventeen mice challenged showed sterile immunity (as defined by the absence of patent parasitemia over the following 30 d), compared to 0 of 17 mice in the AdM42 group, $P = 0.0002$ (**Supplementary Table 1a**). Hence, AdM42-C4bp immunization enhances T_H1-type antibody and $CD4^+$ T cell responses, and it not only provides complete protection against blood-stage challenge but also provides a higher quality of protection as defined by the reduced degree of blood-stage parasitemia. The underlying mechanism for the enhanced immunogenicity remains unknown but may include oligomerization of the antigen[9,10], or the ability of the core domain of mouse C4bp alpha chain to bind CD40[12] or C-reactive protein[13]. The latter two ligands may target antigens fused to mC4bp to receptors on antigen-presenting cells.

$MSP\text{-}1_{33}$–specific $CD4^+$ T cells, but not antibodies, have been reported to protect mice against *P. yoelii*[6,14]. To assess the protective contribution of vaccine-induced T cell responses against blood-stage parasite challenge, mice were immunized as before with the most protective regime (AdM42-C4bp) and depleted of $CD8^+$ or $CD4^+$ T cells before pRBC challenge. Depletion of T cells did not affect protective efficacy (**Supplementary Table 1b**). Vaccine-induced antibody responses against $MSP\text{-}1_{42}$ thus seem sufficient to protect mice in this model at the time of challenge, although other non-$CD4^+$ and non-$CD8^+$ cellular responses may still contribute to immunity[15].

Protection against *P. yoelii* blood-stage parasites is under genetic control[16]. To assess whether this immunization strategy can protect mice on a different genetic background, we assessed immunogenicity and efficacy in C57BL/6 mice. Total IgG responses to $MSP\text{-}1_{19}$ and $MSP\text{-}1_{33}$ in the AdM42-C4bp group showed only a marginal, non-significant increase over those in the AdM42 group (**Supplementary Fig. 1a** online). $MSP\text{-}1_{33}$–specific $CD8^+$ IFN-γ^+ T cell responses were measureable in the spleen and, unlike in BALB/c mice, $CD4^+$ IFN-γ^+ T cell responses against both $MSP\text{-}1_{33}$ and $MSP\text{-}1_{19}$ were detectable (**Supplementary Fig. 1b**). The $CD4^+$ IFN-γ^+ T cell response against $MSP\text{-}1_{33}$, but not $MSP\text{-}1_{19}$, was significantly stronger when using the $MSP\text{-}1_{42}$–C4bp construct, and the $CD8^+$ IFN-γ^+ T cell response to $MSP\text{-}1_{33}$ was enhanced a notable threefold (**Supplementary Fig. 1b**). All of the immunized mice were protected against challenge with pRBCs (**Supplementary Table 1c**), with those mice in the AdM42-C4bp group again showing a higher degree of protection compared to the AdM42 group. These data indicate that the efficacy of AdM42-C4bp immunization is not restricted to one genetic background, and that an equivalent strategy targeting *P. falciparum* MSP-1 may be efficacious in a genetically diverse human population.

To confirm whether AdHu5-MVA immunization can induce functional growth inhibitory antibodies against the human malaria parasite *P. falciparum*, we developed vectors expressing a new *P. falciparum* MSP-1 vaccine construct termed PfM128 that includes both dimorphic sequences of $PfMSP\text{-}1_{42}$ (**Supplementary Methods**). Serum from ten BALB/c mice, immunized as before with AdHu5-PfM128 and boosted with 1×10^7 plaque-forming units MVA-PfM128, was pooled 2 weeks after boost. Mouse IgG was purified, and high-titer responses to both variants of $MSP\text{-}1_{42}$ were identified on a standardized ELISA—endpoints of 181,291 against the 3D7 variant and 153,354 against the FVO variant. Of note, the purified IgG also yielded strong GIA in a standardized *in vitro* assay against diverse *P. falciparum* strains—70% inhibition against 3D7 and 85% against FVO. Purified control mouse IgG showed no significant biological activity against either parasite strain.

The field of blood-stage malaria vaccine development has struggled with the difficulty of achieving substantial protection with human-compatible adjuvants in most preclinical models. We demonstrate that antibody-mediated blood-stage immunity against malaria can be induced in an animal model with only two immunizations, and this is achievable with adjuvant-free viral vectors. This regime is highly immunogenic for MSP-1–specific cellular responses but also unexpectedly immunogenic for antibody induction. Comparable protection has only been achieved with multiple doses of protein-based vaccines, usually with adjuvants that are unsuitable for human use[6,7]. This approach has major implications for extending viral vector–based immunization strategies to numerous other pathogens for which both T cells and antibodies are required for protection.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

S.J.D. designed and made the viruses, performed the *in vivo* experiments and wrote the manuscript. A.C.M. supervised the *in vivo* experiments. A.L.G. assisted with the GIA experiment. C.A.L. performed the GIA assay and standardized the ELISA. A.A.H. advised on the design of the PfM128 vaccine construct. S.C.G. supervised the design of the viruses. F.H. identified the adjuvant effect of mC4bp for protein vaccines and provided the $MSP\text{-}1_{42}$–C4bp construct. A.V.S.H. supervised the experiments and contributed to the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemedicine/.

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