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Short Communication

# Short-term, but not post-exposure, protection against lethal orthopoxvirus challenge after immunization with modified vaccinia virus Ankara

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Safety-tested vaccinia virus (VACV) MVA serves as a candidate third-generation vaccine against smallpox. Here, MVA immunization of mice shortly before or after lethal respiratory challenge with VACV Western Reserve was investigated. Whilst post-exposure treatment failed to protect animals, immunizations on day 2 prior to challenge were fully protective. On the day of challenge, MVA inoculation may prevent death, but not onset of severe respiratory disease. After intranasal MVA application, massive influx of leukocytes (such as neutrophils, macrophages, natural killer cells and T cells) was found in the lungs of the animals, indicating the contribution of innate responses to protection. Correspondingly, in RAG-$1^{-/-}$ mice, MVA inoculation delayed onset of disease significantly, but did not prevent fatal infection. Thus, short-term protection required a tight interplay of both innate and adaptive antiviral immunity. These data suggest that, in addition to conventional vaccination, MVA may serve for potent emergency prophylaxis against orthopoxvirus infection.

In 1980, the World Health Organization declared the eradication of free-living *Variola virus* as the formidable outcome of a unique worldwide smallpox-vaccination campaign. In response to this success, the use of *Vaccinia virus* (VACV) as a live virus vaccine declined and orthopoxvirus-specific vaccines lacked years in product development in comparison with other vaccines. At present, great efforts are being made to be prepared for a potential malevolent use of *Variola virus* or other human-pathogenic orthopoxviruses causing smallpox-like disease (Harrison *et al.*, 2004). Because of safety considerations, highly attenuated and replication-deficient modified VACV Ankara (MVA) holds great promise to replace conventional vaccines based on fully replication-competent VACV (Rosenthal *et al.*, 2001). When compared with the licensed smallpox vaccine Dryvax, MVA immunizations have been shown to elicit equal levels of humoral or cellular immunity and to protect efficiently against lethal orthopoxvirus challenges in mice and non-human primates (Drexler *et al.*, 2003; Earl *et al.*, 2004; Meseda *et al.*, 2005; Stittelaar *et al.*, 2005; Sutter & Staib, 2003; Wyatt *et al.*, 2004). Whilst MVA is being actively developed as a safe, third-generation smallpox vaccine in the USA, costs for a population-wide prophylactic vaccination will probably be substantially higher than those associated with immunization with standard VACV. Further, it remains unclear how often and at what intervals revaccinations would be required to maintain necessary levels of immunity in the population. It should be of advantage to have efficacious vaccines readily available right at the moment of a potential bioterroristic attack. Here, we evaluated the suitability of MVA for vaccinations close to exposure time in the well-established model system of respiratory infection of mice with virulent VACV strain Western Reserve (VACV WR). In contrast to vaccination with conventional VACV strain Elstree, we found substantial efficacy of MVA immunizations at short times before challenge infection. Both innate and adaptive immune responses appeared important to allow for protection against severe disease or death. Post-exposure vaccinations, however, did not markedly influence the outcome of challenge.

We decided to investigate MVA vaccination in the mouse pneumonia model because the respiratory tract is considered as the most relevant entrance route for pathogenic orthopoxviruses. To prevent this infection, vaccination by the intranasal route might be particularly suitable and represents an interesting approach in the development of new orthopoxvirus-specific vaccines. First, we wished to confirm that immunization by the respiratory route can induce appropriate antiviral immunity and tested the potential of intranasal MVA vaccination. Very similar to previous findings for prophylactic intramuscular MVA vaccination

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in this mouse model (Drexler *et al.*, 2003; Staib *et al.*, 2005; Wyatt *et al.*, 2004), we observed full protective capacity of intranasal immunization with $10^8$ infectious units (i.u.) MVA vaccine against challenge infection with a lethal dose of $10^6$ p.f.u. VACV WR diluted in 30 µl PBS [corresponding to about 20 lethal doses 50 ($LD_{50}$)] 3 months after vaccination (Fig. 1a). All animal experiments were performed at least twice and animals were anaesthetized before intranasal inoculation of MVA vaccine or VACV WR challenge infection. Body weight was monitored daily in individual animals and is expressed as the mean for each group.

In order to compare the protective efficacy of intranasal and intramuscular immunization directly and to evaluate the potential usefulness of vaccinations in a close time lag to the exposure of challenge virus, we vaccinated mice with $10^8$ i.u. MVA vaccine and applied a respiratory-challenge infection, inoculating $5 \times 10^4$ p.f.u. diluted in 30 µl PBS (about 1 $LD_{50}$) of VACV WR 2 days after MVA inoculation. Interestingly, both intranasal and intramuscular vaccination protected animals in a nearly equal manner from severe disease or death (Fig. 1b). In contrast, all mock-vaccinated animals showed grave symptoms of illness (data not shown)

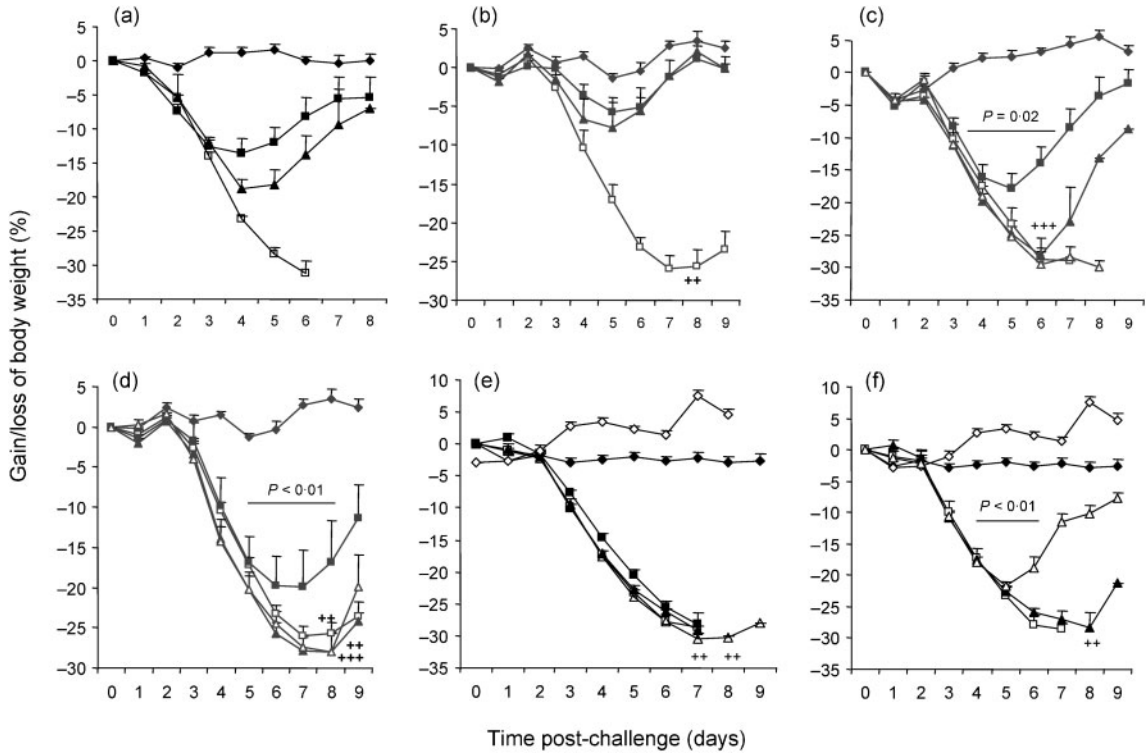


**Fig. 1.** Prophylactic and post-exposure vaccination and protection from VACV WR challenge. (a) BALB/c mice (6 weeks old, $n=4$) were vaccinated intranasally (■) or intramuscularly (▲) with $10^8$ i.u. MVA and submitted to a respiratory challenge with $10^6$ p.f.u. VACV WR 3 months after immunization. Mock-vaccinated (□) and mock-challenged (◆) mice served as control groups. (b) BALB/c mice (6–8 weeks old, $n=5$) were vaccinated intranasally (■) or intramuscularly (▲) with $10^8$ i.u. MVA and, after 2 days, challenged intranasally with $5 \times 10^4$ p.f.u. VACV WR. Mock-vaccinated (□) and mock-challenged (◆) mice served as control groups. (+) indicates animals that died or had to be sacrificed. (c) BALB/c mice (6–8 weeks old, $n=4$) were vaccinated intranasally with $10^5$ (△), $10^6$ (▲) or $10^7$ (■) i.u. MVA. After 2 days, mice were challenged intranasally with $10^6$ p.f.u. VACV WR. Mock-vaccinated (□) and mock-challenged (◆) mice served as control groups. (+) indicates animals that died or had to be sacrificed. (d) BALB/c mice (6–8 weeks old, $n=5$) were challenged intranasally with $5 \times 10^4$ p.f.u. WR and vaccinated intramuscularly with $10^8$ i.u. MVA on the same day (■), on day 1 (▲) or on day 2 (△) after exposure. Mock-vaccinated (□) and mock-challenged (◆) mice served as control groups. It should be noted that the same group of mock-challenged/mock-vaccinated animals as shown in panel (b) served as controls in this experiment. (e) BALB/c mice (6–8 weeks old, $n=5$) were immunized with VACV Elstree by scarification at 2 days prior to (△), on the same day as (■) or on day 1 after (▲) challenge with $10^6$ p.f.u. VACV WR. (f) BALB/c mice (6–8 weeks old, $n=5$) were vaccinated intramuscularly 2 days prior to challenge with $10^6$ p.f.u. VACV Elstree (▲) or with $10^8$ i.u. MVA (△). Mock-vaccinated mice (□), mock-challenged mice (◆) and mice that were immunized by scarification with VACV Elstree 2 weeks prior to challenge (◇) served as control groups for the parallel experiments shown in panels (e) and (f). (+) indicates animals that died or had to be sacrificed. Standard errors are indicated as SEM; *P* values indicate a significant difference from other vaccine groups as determined by Student's *t*-test.

and two animals did not survive the infection. Importantly, these data are a first proof of principle for the feasibility of protective vaccination at short times before infection. Moreover, we vaccinated mice, again intranasally, with decreasing doses of $10^7$, $10^6$ or $10^5$ i.u. MVA vaccine 2 days before applying a high-dose ($10^6$ p.f.u.) VACV WR challenge infection. This time, the inoculation of VACV WR resulted in the onset of respiratory disease in all animals, and most mice from the groups receiving $10^5$ or $10^6$ i.u. MVA died from infection or had to be sacrificed. Yet, in the group being vaccinated with the highest dose of MVA ($10^7$ i.u.), weight loss was reduced significantly ($P=0{\cdot}02$ compared with the group receiving $10^6$ i.u.) and all animals survived the challenge (Fig. 1c). These data showed, in principle, the suitability of short-term vaccination to also achieve protection against a harsh respiratory-challenge infection. Moreover, the level of protection appeared comparable to the efficacy of conventional MVA immunizations in the context of a challenge infection in the immune-memory phase, e.g. at 3 months after vaccination (Staib *et al.*, 2005; Fig. 1a).

In the next series of experiments, we wished to mimic a scenario of post-exposure vaccination. There exists anecdotal information from which it is suggested that prompt vaccination of individuals exposed to *Variola virus* might have prevented severe smallpox disease (Fenner *et al.*, 1988; Mortimer, 2003). In our experiments, however, the protective capacity of intramuscular MVA immunization ($10^8$ i.u.) proved to be reduced severely when given after the respiratory infection with $5\times10^4$ p.f.u. VACV WR (Fig. 1d). Only inoculations of MVA vaccine within 3 h of challenge resulted in significant protection of all animals ($P<0{\cdot}01$ in comparison with other groups receiving challenge infections), but did not prevent the onset of substantial disease symptoms (data not shown). Moreover, MVA immunizations applied 1–4 days after challenge infection had no effect compared with mock vaccination (Fig. 1d; data not shown). Yet, the question arose whether standard vaccines based on replication-competent VACV would protect better than immunizations with the non-replicating MVA vaccine. Thus, in an additional experiment, we used a vaccine based on VACV strain Elstree, which originated from a German vaccine stock prepared for human vaccination by scarification, and tested its efficacy in short-term prophylaxis and post-exposure treatment. Groups of mice were vaccinated with $10^6$ p.f.u. Elstree vaccine by scarification 2 days prior to or on days 0, +1 or +2 after respiratory-challenge infection with $10^6$ p.f.u. VACV WR (Fig. 1e; data not shown). All Elstree immunizations given 2 days before or at any time after challenge failed to protect against lethal disease. However, control animals immunized by conventional scarification (Elstree vaccine given 14 days prior to challenge) were protected solidly, showing the typical efficacy of prophylactic vaccination with replicating VACV. The lacking efficacy of VACV Elstree vaccine used at times near to challenge infection could be attributed to the route of scarification. Therefore, despite the fact that vaccines based on replication-competent VACV are not considered suitable for systemic immunizations, we also tested the possibility for enhanced efficacy of intramuscular vaccination with $10^6$ p.f.u. VACV Elstree on day 2 prior to challenge. This dosage of VACV Elstree vaccine seemed appropriate because, in previous experiments for prophylactic intramuscular vaccination in the mouse model, we had found $10^5$–$10^6$ p.f.u. replication-competent VACV Wyeth vaccine to be equally immunogenic and protective as $10^7$–$10^8$ i.u. MVA vaccine (Drexler *et al.*, 2003). Yet, we again failed to observe short-term protective capacity of the VACV Elstree immunizations (Fig. 1f). In contrast, intramuscular inoculations of $10^8$ i.u. MVA vaccine again protected all animals significantly better against the harsh challenge infection ($P<0{\cdot}01$ compared with the group receiving VACV Elstree), still resulting in loss of body weight (Fig. 1f), but causing only minor signs of illness (data not shown). The data from this experiment clearly confirmed the particular efficacy of MVA immunizations when applied shortly before challenge. However, our overall data also suggest that the practicability of post-exposure vaccination against smallpox might be limited, at least in the context of naïve individuals or a harsh respiratory infection. This assumption is corroborated by the recent finding of limited efficacy of smallpox vaccination given 24 h after a lethal intratracheal infection of cynomolgus macaques with *Monkeypox virus* (Stittelaar *et al.*, 2006). Moreover, past sources of information on post-exposure vaccination seem to indicate that it could be mainly revaccination of previously immunized individuals that successfully prevented smallpox (Mortimer, 2003). Modalities other than vaccination could be more suitable for post-exposure treatment, as suggested by the use of antiviral drugs in the macaque–monkeypox model (Stittelaar *et al.*, 2006) or the therapeutic application of VACV-specific antibodies, including those directed against extracellular forms of virus, in the BALB/c mouse pneumonia model (Law *et al.*, 2005).

On the other hand, our data provide first evidence for the possibility of short-term immunizations against orthopoxvirus infections. The development of MVA as a candidate vaccine against pathogenic orthopoxvirus infections was based on encouraging data from testing conventional prophylactic MVA vaccination in mouse and non-human primate challenge models. Now, stockpiling of an effective and safe MVA emergency vaccine could be an attractive alternative measure against the potential use of orthopoxviruses as biological weapon. Interestingly, immunizations by the respiratory or the intramuscular route provided very similar levels of short-term protection. This finding was somewhat surprising, as we had speculated on a possible advantage of intranasal vaccine delivery, e.g. mediating particular protective efficacy through local innate responses. The lacking efficacy of the VACV Elstree vaccine might be explained by the need of replication-competent viruses for sufficient time for antigen amplification or by their lesser immunostimulatory capacity, due to conserved viral

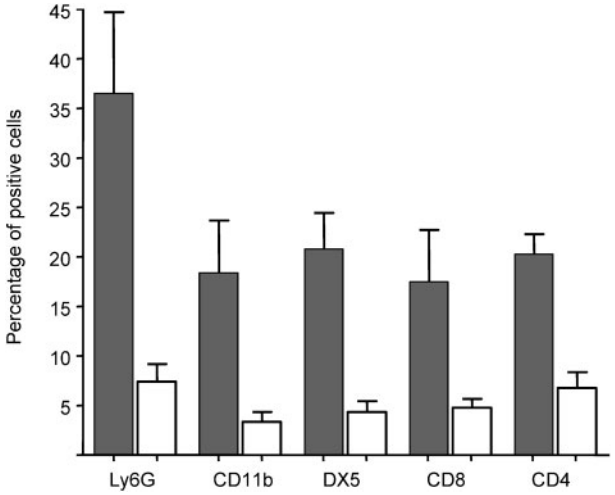


**Fig. 2.** Bronchoalveolar lavage of BALB/c mice ($n=4$) inoculated intranasally with $10^8$ i.u. MVA or Tris-buffered saline. Shaded bars, MVA; empty bars, Tris-buffered saline. Mean percentages of living marker-positive cells are shown.

mechanisms counteracting the innate immune system. Thus, the immunological correlates providing the mechanistic basis for short-term efficacy of MVA immunization could be complex and still need to be elucidated in more detail. In first experiments, we determined by bronchoalveolar lavage a massive influx of immune cells into the lungs of mice within 48 h of intranasal MVA vaccine application. Isolated cells were stained with propidium iodide, anti-Ly6G–phycoerythrin (PE) (used for detection of granulocytes, i.e. neutrophils), anti-CD11b–PE–Cy7 (for detection of myeloid cells including macrophages), anti-DX5–fluorescein isothiocyanate (for detection of natural killer cells and some T-cell subsets), anti-CD8–PE–Cy5 and anti-CD4–PE–Texas red, followed by six-colour fluorescence-associated cell-sorting analysis using a CyAn cytometer (DakoCytomation) and Summit (v. 4.0) software. Compared with saline inoculation, MVA instillation induced the invasion of dramatically higher numbers of Ly6G-, CD11b-, CD49b/DX5-positive leukocytes, such as neutrophils, macrophages and natural killer cells, concurrently with clearly elevated levels of $CD8^+$ and $CD4^+$ T cells (Fig. 2). Moreover, MVA inoculation in RAG-$1^{-/-}$ mice being compromised in adaptive immune responses (Mombaerts *et al.*, 1992) delayed the onset of severe respiratory disease significantly ($P<0{\cdot}001$ compared with mock-vaccinated animals) (Fig. 3a) but, in contrast to vaccination of fully immune-competent mice (Fig. 3b), did not prevent fatal outcome of infection. Thus, the short-term protection against lethal orthopoxvirus infection observed after MVA inoculation is obviously based on an intimate interplay between innate and adaptive antiviral immunity. Indeed, a number of previous studies have indicated the capacity of MVA to stimulate the migratory or phagocytic activity of immune cells (Förster *et al.*, 1994), to induce type I interferon production (Blanchard *et al.*, 1998; Hornemann *et al.*, 2003) or to activate the NF-$\kappa$B response pathways in infected cells (Oie & Pickup, 2001). Additional studies, possibly in various model systems, will be needed to carefully dissect the effectors of different immune responses and to determine their relative contribution.

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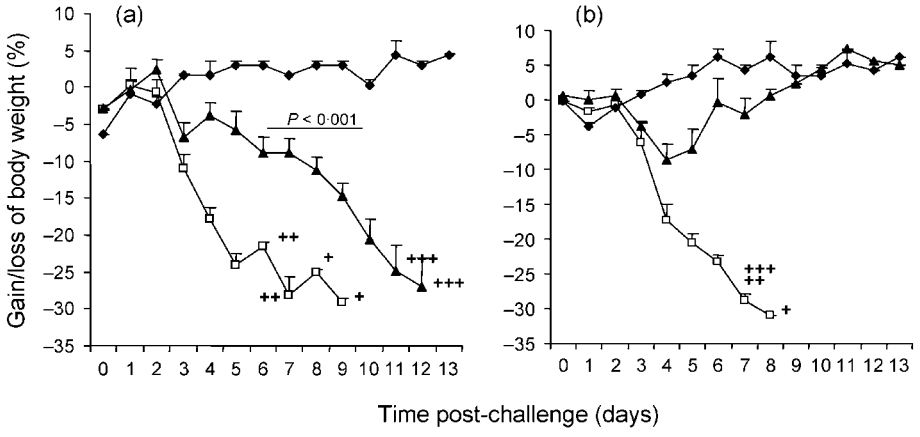


**Fig. 3.** Pre-exposure vaccination of B- and T-cell-deficient mice. (a) RAG-$1^{-/-}$ mice (8–12 weeks old, $n=6$) or (b) C57BL/6 mice (6–8 weeks old, $n=6$) were vaccinated intranasally with $10^8$ (▲) i.u. MVA. After 2 days, mice were challenged intranasally with $5\times10^5$ p.f.u. VACV WR. Mock-vaccinated (□) and mock-challenged (◆) mice served as control groups. (+) indicates animals that died or had to be sacrificed. *P* values indicate a significant difference from other vaccine groups as determined by Student's *t*-test.

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