

RESEARCH ARTICLE

Deletion of Fifteen Open Reading Frames from Modified Vaccinia Virus Ankara Fails to Improve Immunogenicity

Naif Khalaf Alharbi^{1,2*}, Alexandra J. Spencer¹, Adrian V. S. Hill¹, Sarah C. Gilbert¹

1 The Jenner Institute, University of Oxford, Oxford, OX3 7DQ, United Kingdom, **2** King Abdullah International Medical Research Center, Riyadh, Saudi Arabia

* naif.alharbi@ndm.ox.ac.uk



OPEN ACCESS

Citation: Alharbi NK, Spencer AJ, Hill AVS, Gilbert SC (2015) Deletion of Fifteen Open Reading Frames from Modified Vaccinia Virus Ankara Fails to Improve Immunogenicity. PLoS ONE 10(6): e0128626. doi:10.1371/journal.pone.0128626

Academic Editor: Steven M. Varga, University of Iowa, UNITED STATES

Received: January 21, 2015

Accepted: April 29, 2015

Published: June 8, 2015

Copyright: © 2015 Alharbi et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper.

Funding: This work was funded in part by a Grand Challenges in Global Health grant to Prof. Adrian V. S. Hill, Jenner Institute, University of Oxford administered by the Foundation for the National Institutes of Health. AJS is a Oxford Martin Fellow and SCG and AVS are Jenner Institute Investigators (<http://www.jenner.ac.uk>). NKA receives a scholarship fund from King Abdullah International Research Centre, Riyadh, Saudi Arabia (<http://www.kaimrc.med.sa>). The funders had no role in study design,

Abstract

Modified vaccinia virus Ankara (MVA) is a highly attenuated strain of vaccinia virus, which has been used as a recombinant vaccine vector in many vaccine development programmes. The loss of many immunosuppressive and host-range genes resulted in a safe and immunogenic vaccine vector. However it still retains some immunomodulatory genes that may reduce MVA immunogenicity. Earlier reports demonstrated that the deletion of the *A41L*, *B15R*, *C6L*, or *C12L* open reading frames (ORFs) enhanced cellular immune responses in recombinant MVA (rMVA) by up to 2-fold. However, previously, we showed that deletion of the *C12L*, *A44L*, *A46R*, *B7R*, or *B15R* ORFs from rMVA, using MVA-BAC recombineering technology, did not enhance rMVA immunogenicity at either peak or memory cellular immune responses. Here, we extend our previous study to examine the effect of deleting clusters of genes on rMVA cellular immunogenicity. Two clusters of fifteen genes were deleted in one rMVA mutant that encodes either the 85A antigen of *Mycobacterium tuberculosis* or an immunodominant H2-K^d-restricted murine malaria epitope (pb9). The deletion mutants were tested in prime only or prime and boost vaccination regimens. The responses showed no improved peak or memory CD8⁺ T cell frequencies. Our results suggest that the reported small increases in MVA deletion mutants could not be replicated with different antigens, or epitopes. Therefore, the gene deletion strategy may not be taken as a generic approach for improving the immunogenicity of MVA-based vaccines, and should be carefully assessed for every individual recombinant antigen.

Introduction

Modified Vaccinia virus Ankara (MVA) is a highly attenuated strain of vaccinia virus, the smallpox vaccine. This attenuation was achieved by more than five hundred passages in chick embryo fibroblast (CEF) cells [1]. The resultant MVA is incapable of replication in almost all tested mammalian cells, except the baby hamster kidney cell line (BHK-21) [2]. MVA is safe virus that was used to vaccinate 120000 people during the WHO smallpox eradication initiative

data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: SCG and AVSH are named inventors on patents and patent applications relating to prime-boost vaccination. MVA flu vaccine: EP2044947 (A1) Compositions and methods; US2010285050 (A1) Compositions and methods. Gene Insertion into MVA: WO 2011128704 A1 Pox virus expression system. There are no further patents, products in development or marketed products to declare. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

in 1970s without adverse events [3]. The first clinical use of recombinant MVA (rMVA) as a vaccine against a human pathogen was in 2000. The safety profiles of the recombinant vaccine were as expected with a replication-deficient virus, and no live or dead virus (screened by PCR) could be detected in samples from the site of inoculation [4]. In the last decade, MVA-based vaccines have been tested in an increasing range of animal models and many clinical trials for vaccines against malaria, HIV/AIDS, Influenza, TB [5–7], and now Ebola (manuscript in preparation). The attenuation of MVA resulted in the loss of almost a third of its parental genome, deleting or mutating the majority of immune evasion and host range genes [8]. By losing immunomodulatory genes, MVA has become a strong immune activator, infecting a wide range of immune cells and eliciting a greater range of chemokines and cytokines compared to the parental vaccinia virus [9, 10]. However, it has retained some genes that are involved in the host-virus interaction and immune evasion such as *B15R*, encoding the IL-1 β binding protein [11], or *A41L*, encoding a CC-chemokine binding protein [12].

It has been reported that deletion of some of these genes improved innate, adaptive memory cellular or humoral immune responses to the vector (MVA) or to the encoded recombinant antigens, and enhanced the protective capacity of MVA following a challenge with VACV WR. The deletion of the ORF *A41L* [12], *B15R* [11], or both [13], showed around 2-fold increase in the memory CD8⁺ T cell responses upon stimulation with VACV-infected cells or MVA-specific peptides. The immune responses to the recombinant antigens were also increased to a similar extent. In the double deletion $\Delta A41L/B15R$ [13], the MVA vector encoded HIV-1 clade B immunogen (MVA-B), and was delivered by DNA-prime MVA-boost immunization. The use of different peptide pools to stimulate the splenocytes from vaccinated mice revealed an increased CD8⁺ T cell response to one of the pools (GPN-pool) in the rMVA mutant vaccinated group. This mutant also induced more CD8⁺ T cells than CD4⁺ T cells in the Env-pool. In another study, the deletion of *C6L* gene from MVA-B ($\Delta C6$ -MVA-B) increased memory CD4⁺ and CD8⁺ T cell responses, and although this mutant did not shift the immune responses towards particular peptide pools, the increased CD4⁺ or CD8⁺ T cells were only observed in one peptide pool (the GPN-pool), but not in the Env- or Gag-pool [14]. Although the $\Delta C6$ -MVA-B was not tested for the peak (acute phase) adaptive immunity, a recent study from the same research group testing the effect of a double-deletion mutant lacking *C6L* and *K7R* in the same MVA-B vaccine ($\Delta C6/K7$ -MVA-B) showed that the $\Delta C6$ -MVA-B, included as a control, had no impact on the peak immune responses [15]. This is perhaps not surprising as the majority of the previous studies reported small (around 2-fold) or no enhanced immunogenicity of MVA deletion mutants at the acute adaptive phase. The double-deletion $\Delta C6/K7$ -MVA-B showed increased memory CD8⁺ T cells, but not CD4⁺ T cells; and upon comparison with the $\Delta C6$ -MVA-B, the increase seemed to be mainly an effect of *C6* absence and not due to the *K7R* deletion [15]. Furthermore, in more recent studies, increased memory CD8⁺ T cells specific to the MVA vector or to encoded antigens were also reported upon the single deletion of *FIL* [16], *N2L* [17], or *C12L* (MVA008L) [18] in rMVA with around 2-fold increase. Overall, it appears that the increased cellular immunogenicity is antigen-specific, or rather epitope specific, which could make it difficult to employ the same deletion in a different MVA-based vaccine before carefully assessing the immunological outcome, and the detectable increase in immunogenicity is always small.

In terms of improving humoral immunogenicity in these studies, while the deletion of *A41L*, *B15R*, or the double deletion of both had no impact on the antibody induction at the memory phase, the deletion of *C6L* or *N2L* increased the memory antibody-mediated responses by around 2-fold in the total anti-recombinant antigen antibody titres. Of note, the *C6L* deletion enhanced the anti-gp120 antibody titres [14], but the re-testing of the *C6L* deletion mutant, in a later study, along with the double deletion *C6L/K7R* mutant showed no

significant difference in the anti-gp120 antibody titres by those two mutants compared to the control (MVA-B vaccine) [15]. The humoral responses were not shown in the *FIL* and *C12L* studies. Finally, the deletion of *A35R* showed small non-significant increase in the cellular immunogenicity but increased the anti-VACV antibody titres by 2-fold with isotype switching to more IgG and its subclasses IgG1 and IgG2a [19]. Overall, in murine studies, it seems that all reported increases in the immunogenicity of MVA mutants are small, with 2- to 3-fold at most. In these reports there are many important variables such as the dose and route of administration, vaccination regimens (prime only or prime-boost), recombinant antigens, immunological readouts (e.g. ELISpot vs. ICS), mouse strains, time and type of stimuli used in the *ex vivo* re-stimulation (e.g. VACV-infected cells vs. E3 or F2(G) peptides), and the time for harvesting the spleens to determine memory responses (varies from 10, 21, 56, or 180 days post-MVA injection).

Nevertheless, a study in macaques reported that deleting four genes; *C12L* (MVA008L), *B15R* (MVA184R), *A41L* (MVA153L), and *A46R* (MVA159R) from MVA encoding HIV-1 clade C consensus Gag and Env immunogen, given twice with a 9 week interval, resulted in increased CD4⁺ and CD8⁺ T cells either after the prime (4-fold) or after the boost dose (5-fold); and around 25-fold increase in the anti-gp120 antibody titres [20]. In this report, the deletion of uracil-DNA-glycosylate (*udg*) gene, in addition to those four genes, resulted in a five-gene deletion rMVA mutant that did not further enhance the improved immunogenicity of the four-gene deletion mutant. The deletion of the fifth gene, *udg*, was expected to improve the immune responses, as a previous report by the same group showed that the deletion of *udg* gene alone in rMVA inhibited the late MVA gene expression, reduced the antigen complexity of MVA, and improved the elicited CD4⁺ and CD8⁺ T cells (2-fold) in a similar regimen, but with Gag gene from HIV clade B, which is a different recombinant antigen [21].

Previously, we reported that the single deletion of *C12L*, *A44L*, *A46R*, *B7R*, or *B15R*, from MVA did not enhance the cellular immunogenicity of the TIP model antigen (described in material and methods) using seven immunodominant MVA epitopes in two strains of mice at the peak cellular responses. Of the memory responses (day 56 post-MVA injection), only the Δ B15R-MVA showed a small (1.5-fold) but significant increase in the CD8⁺ T cell frequencies [22]. Here, we have extended our previous study using MVA-BAC recombineering technology to examine the effect of deleting clusters of genes (described in Table 1), including a large deletion of 15 genes in one mutant, on the cellular immunogenicity of rMVA. These deleted genes have different immunomodulatory functions or, in some instances, unknown functions. The derived recombinant MVA mutants (rMVA mutants) encode either the 85A antigen of *M. tuberculosis* [23] or the TIP model epitope string-based antigen [24] that encompasses an immune dominant H2-K^d-restricted murine malaria epitope (pb9) from the *Plasmodium berghei* circumsporozoite protein [25]. The MVA-BAC system was shown previously to have no altering effect on the immunogenicity of recombinant MVA, and elicit anti-vector immunogenicity similar to the conventionally derived recombinant MVA [22].

None of the derived rMVA mutants showed improved immunogenicity either to the MVA or to encoded TIP antigens. The large MVA deletion mutant (lacking fifteen genes) was tested in prime only or in prime-boost regimens and showed no improved CD8⁺ T cell frequencies. This unchanged immunogenicity was observed using either the synthetic model TIP antigen or the naturally occurring 85A antigen. The responses at 28, 56, or 84 (long-term) days post-MVA injection showed no improved memory CD8⁺ T cell frequencies, except to one peptide (E3) at day 28. However, at day 84, or at day 56 in the prime-boost vaccination, this increase in E3-specific responses was very marginal and not statistically significant. This support the earlier studies showing that the increase could be observed with only few peptides or peptide pools.

Table 1. MVA deletion mutants.

MVA	Deleted ORF / Function (Reference)	Note
MVAwt	None	Not recombinant virus. Not modified virus. Used as a control.
MVA85A	None	Conventionally derived rMVA with 85A at the TK ^a . Used as a control for 85A responses.
MVA-BAC-85A	None	BAC recombineering-derived rMVA with 85A at the TK ^a locus. Contains BAC DNA and expresses GFP. Used as a control for 85A responses.
MVA-TIP	None	Contains BAC DNA, expresses GFP and encodes TIP antigen at TK ^a locus. Used as a control for TIP responses.
ΔC11-K3/B15-MVA	<i>C11R</i> / viral growth factor [32]. <i>C10L</i> / suggested IL-1 binding protein [33]. <i>MVA008R</i> , not in VACV COP ^b , but <i>C12L</i> in WR ^c / IL-18 binding protein [34]. <i>D7L</i> / RNA polymerase subunit [35]. <i>MVA007R</i> , and <i>009L-013L</i> (fragmented, not in VACV COP ^b) / cowpox host range genes [8]. <i>C9L</i> / (fragmented gene) [8]. <i>C8L</i> / unknown. <i>C7L</i> / human cells host range gene [36, 37]. <i>C6L</i> / anti-IFN-β activation (IRF3/IRF7 inhibitor and Bcl-2 family) [38]. <i>N1L</i> / anti-apoptotic (Bcl-2 family) [39]. <i>N2L</i> / anti-IFN-β activation (IRF3 inhibitor and Bcl-2 family) [40]. <i>K1L</i> (inactive gene) / human cells host range gene [41]. <i>K2L</i> / cell fusion (serine protease) inhibitor [42]. <i>K3L</i> / anti-apoptotic (PKR inhibitor) [43]. <i>B15R^d</i> / IL-1β binding protein [11].	Contains BAC DNA, expresses GFP, and encodes TIP antigen at TK.
ΔB7-15-MVA	<i>B7R</i> / TNF-α soluble receptor and chemokine binding protein [44]. <i>B8R</i> (fragmented) / IFN-γ soluble receptor [45]. <i>B9R</i> / intracellular protein [46]. <i>B10R</i> / unknown. <i>B11R</i> / unknown. <i>B12R</i> / serine/threonine protein kinase [47]. <i>B13R</i> + <i>14R</i> (fragmented). <i>B15R^d</i> / IL-1β binding protein [11].	Contains BAC DNA, expresses GFP, and encodes TIP antigen at TK.
ΔA41-44/B15-MVA	<i>A41L</i> / CC-Chemokine binding protein [12]. <i>A42R</i> / Profilin homolog [48]. <i>A43R</i> / membrane protein [49]. <i>SalF6R</i> (<i>MVA156R</i>) / membrane glycoprotein [50, 51]. <i>A44L</i> / Hydroxysteroid dehydrogenase [51]. <i>B15R^d</i> / IL-1β binding protein [11].	Contains BAC DNA, expresses GFP, and encodes TIP antigen at TK.
ΔA41-46/B15-MVA	Same as above, in addition to <i>A45R</i> / Inactive superoxide dismutase [52] and <i>A46R</i> / TLR signalling inhibitor [53].	Contains BAC DNA, expresses GFP, and encodes TIP antigen at TK.
Δ15-MVA-TIP and Δ15-MVA-85A (i.e. ΔA41L to 46R & B7R to B15R)	<i>A41L</i> , <i>A42R</i> , <i>A43R</i> , <i>A44L</i> , <i>A45R</i> , <i>A46R</i> , <i>B7R</i> , <i>B8R</i> , <i>B9R</i> , <i>B10R</i> , <i>B11R</i> , <i>B12R</i> , <i>B13R</i> , <i>B14R</i> , and <i>B15R^d</i> . In addition to <i>SalF6R</i> , which is located between <i>A42R</i> and <i>A43R</i> . All mentioned above.	Contains BAC DNA, expresses GFP, and encodes TIP antigen at TK.

MVA deletion mutants used in this study. Different clusters of ORFs were deleted from the mutants, encoding proteins with different functions (mentioned with references). In a few cases, there were no known functions associated with ORFs. The mutants express the 85A or TIP antigens that were inserted at the TK locus. All mutants were made using MVA-BAC recombineering, therefore, all contain BAC DNA and GFP (green fluorescent protein) marker.

^aTK: Thymidine kinase locus, used as an insertion site for the recombinant antigens.

^bVACV COP: Vaccinia virus Copenhagen strain.

^cWR: Vaccinia virus Western Reserve strain.

^dThe ORF *B15R* is the *MVA184R* gene. It is named *B15R* in VACV WR while it is *B16R* in VACV COP. This made inconsistency in reporting this ORF as well as the downstream B fragment ORFs in the literature. Here, we report *MVA184* as the *B15R*, consistent with our previous work [22], and in accordance with another report [11]. However, it was reported as *B16R* in another study [13]. This ORF encodes IL-1β binding protein [11].

doi:10.1371/journal.pone.0128626.t001

Our results suggest that the reported increases in MVA deletion mutants, which were small increases in many cases (around 2-fold), could not be replicated with different antigens and that the approach of gene deletion to improve MVA-vectored vaccines should be carefully

assessed for every recombinant antigen rather than taken as a generic approach for improving MVA immunogenicity.

Results

The immunogenicity of recombinant MVA mutants with TIP antigen

To investigate the effect of deleting clusters of genes on the immunogenicity of a recombinant MVA, expressing TIP antigen, we immunized BALB/c mice with the recombinant MVA control (MVA-TIP) or with different recombinant MVA mutants (Δ B7-15-MVA, Δ A41-44/B15-MVA, Δ A41-46/B15-MVA, and Δ C11-K3/B15-MVA, from which a number of ORFs had been deleted, described in Table 1) intradermally (i.d.) and harvested spleens a week later. The IFN- γ -secreting CD8⁺ T cells, specific to MVA-peptides (E3 and F2(G)) or to the TIP-specific peptide (pb9) were then measured. There were no significant differences in response to the MVA-E3, MVA-F2(G), or pb9 peptide between any of the mutant groups and the control (Fig 1).

The construction and sequence screening of a recombinant MVA mutant lacking fifteen genes

As there was no improved immunogenicity with various deletion mutants, we constructed a large MVA mutant, lacking fifteen genes (Δ 15-MVA) described in Table 1, and investigated its impact on the cellular immunogenicity. We made two version of the Δ 15-MVA with either 85A or TIP recombinant antigens (Δ 15-MVA-TIP and Δ 15-MVA-85A). The Δ 15-MVA-85A was based on Δ 15-MVA-TIP and was sent for genomic sequencing. The genome sequencing showed that MVA genome remained unchanged, despite passing through four rounds of MVA-BAC recombineering. Sequencing was performed on BAC DNA as well as on viral DNA, from a BAC-rescued virus, and revealed the presence of the two large deletions (*A41L* to

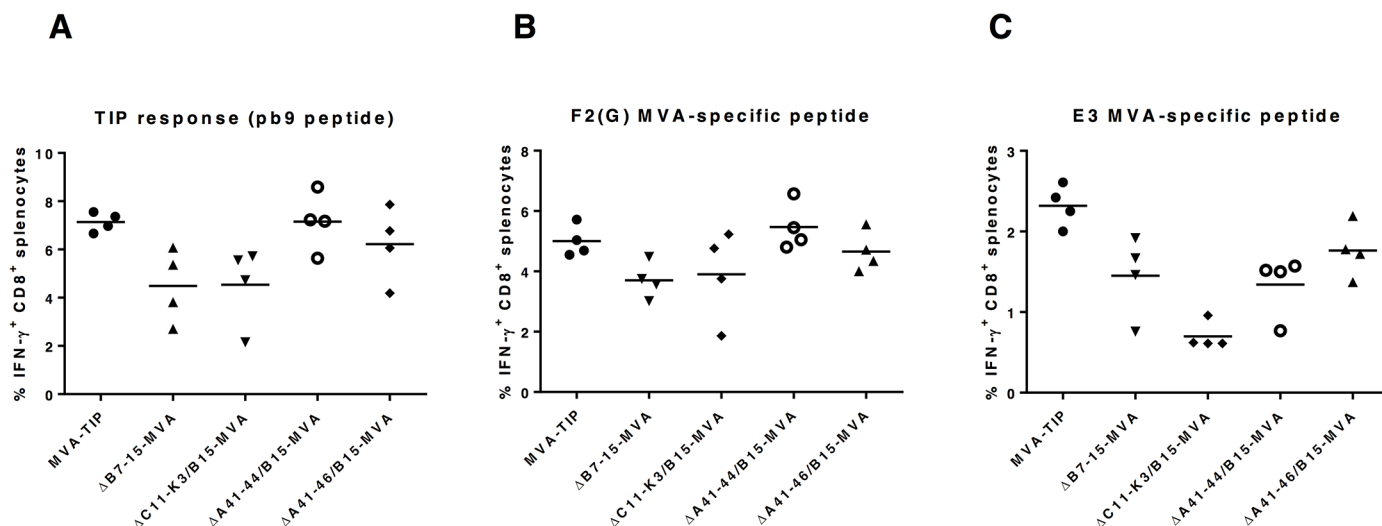


Fig 1. *In vivo* cellular immunogenicity of MVA deletion mutants, lacking clusters of genes, with TIP antigen. Five groups of female BALB/c mice ($n = 4$) were immunized (i.d.) with MVAwt with TIP model antigen (MVA-TIP), or MVA deletion mutant (lacking different clusters of genes, described in Table 1) at the dose of 1×10^6 pfu/ml. **Seven days** post immunization, mice were sacrificed and spleens collected for intracellular cytokine staining and flow cytometry to determine the percentage of IFN- γ -secreting CD8⁺ T splenocytes in response to *in vitro* re-stimulation with pb9 peptide (**A**), or with MVA vector-specific peptides (**B and C**). These values are presented after subtracting the values of unstimulated cells for every mouse (sample). The mean of each group is shown. Data is representative of two independent experiments. There was no statistical significant increase in any of the tested groups as compared to the control (MVA-TIP) using Kruskal-Wallis test with Dunn's multiple comparisons.

doi:10.1371/journal.pone.0128626.g001

A46R and *B7R* to *B15R*). The presence of either 85A or TIP antigens were screened with PCR; and the absence of the MVA wild type was also confirmed by PCR.

The immunogenicity of a recombinant MVA mutant lacking fifteen genes, with TIP antigen

As there was no difference with the TIP antigen responses upon deletion of clusters of genes, we wanted to determine the long-term immune responses of a the new large deletion mutant, $\Delta 15$ -MVA-TIP, measuring the response to pb9, an immunodominant peptide to allow for following the long-term memory immune responses, in addition to measuring the peak immune responses. Ten BALB/c mice, per group, were immunized with either the $\Delta 15$ -MVA-TIP mutant or the control (MVA-TIP) 10^6 pfu via i.m route, and the immune responses followed for three months. First, all mice were bled at day 7 post-immunization and the PBMCs were screened in blood-ELISpot using the pb9 peptide, or the vector-specific E3 and F2(G) peptides. The CD8⁺ T cell immune responses were similar between the MVA-TIP and the mutant ($\Delta 15$ -MVA-TIP). At day 28 post-immunization, five mice from each group were sacrificed, spleen harvested, and ICS was performed using the same three peptides. Only E3-specific CD8⁺ T cell responses were enhanced (in two independent experiments) in the mutant group while there were no differences in the recombinant epitope (pb9) or in the other vector-specific F2(G) peptide. The remaining of the mice were sacrificed at day 84 post-immunization and showed no improved CD8⁺ T cell frequencies, even the increased responses with the E3 peptide at day 28 was not observed at day 84, suggesting it was transient enhancement (Fig 2).

The immunogenicity of a recombinant MVA mutant lacking fifteen genes, with TIP antigen in DNA-prime MVA-boost vaccination

As the fifteen-gene deletion mutant did not show any difference at peak or memory responses, we proceeded to test this mutant in the more clinically relevant prime-boost vaccination regimen. This regimen has been used to test other MVA deletion mutants (e.g. $\Delta A41$ \B15R-MVA-B [13] and $\Delta C6$ -MVA-B [14] with reported increases in immunogenicity from the mutant compared to wild type MVA. Thus, mice were primed with 100 μ g DNA, encoding the TIP antigen, via i.m. route, and boosted two weeks later with 10^7 pfu, via i.p. route, with either the $\Delta 15$ -MVA-TIP mutant or the control (MVA-TIP). Five mice were sacrificed at day 7 post-boost to determine the peak immune responses with the remaining mice sacrificed at day 56 post-boost to determine the memory responses. There were no improved CD8⁺ T cell responses either at the peak or at the memory responses to pb9, E3, or F2(G) peptide stimulations (Fig 3).

The immunogenicity of a recombinant MVA mutant lacking fifteen genes, with 85A antigen

As there was no detectable difference in the immune responses to the TIP, which is a synthetic epitope string-based antigen, we wished to also test a naturally occurring immunogenic antigen. The *M. tuberculosis* Ag85A is being tested as TB vaccine candidate, delivered by MVA vector (MVA85A) and has been shown to induce CD4⁺ and CD8⁺ T cell responses and when used to boost BCG-vaccinated adult or infant humans [23]. Thus, the $\Delta 15$ -MVA-85A mutant along with other three control viruses; MVA wild type (MVA_{wt}), non-mutant MVA-BAC-85A, and the MVA85A (see [materials and methods](#)), were used to immunized BALB/c mice, with 10^6 pfu via i.m route, and the spleens were harvested one week later. The intracellular cytokine staining (ICS) was performed to determine the cellular immune responses using two 85A

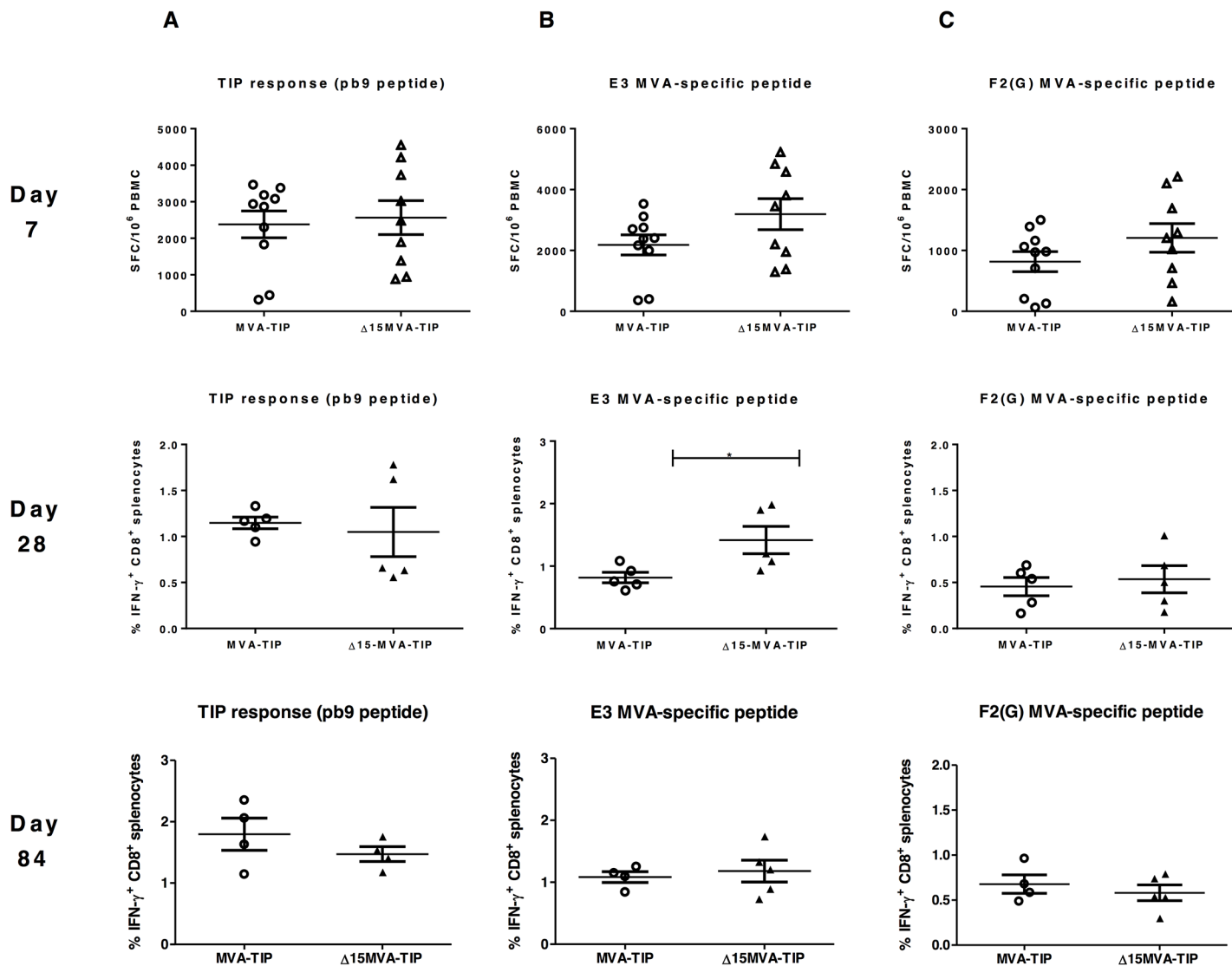


Fig 2. In vivo cellular immunogenicity of MVA deletion mutants, lacking fifteen genes, with TIP antigen. Two groups of female BALB/c mice ($n = 10$) were immunized (i.m.) with MVAwt with TIP model antigen (MVA-TIP), or MVA deletion mutant (lacking 15 genes) $\Delta 15$ -MVA-TIP at the dose of 1×10^6 pfu/ml. **Seven days** post immunization (top), *ex vivo* ELISpot was performed to determine the percentage of IFN- γ -secreting CD8 $^+$ splenocytes in response to *in vitro* re-stimulation with pb9 peptide (A), or with MVA vector-specific peptides (B and C) (all three peptides are CD8 $^+$ T cell specific). **28 days** (middle), or **84 days** (bottom) post immunization, five mice were sacrificed and spleens collected for intracellular cytokine staining and flow cytometry to determine the percentage of IFN- γ -secreting CD8 $^+$ T splenocytes in response to *in vitro* re-stimulation with pb9 peptide (A), or with MVA vector-specific peptides (B and C). These values are presented after subtracting the values of unstimulated cells for every mouse (sample). The mean of each group with the SEM error bars are shown. Data is representative of two independent experiments. * $P = 0.0331$ using Mann Whitney test.

doi:10.1371/journal.pone.0128626.g002

peptide pools, specific for CD4 $^+$ or CD8 $^+$ T cells, or E3 and F2(G) MVA-peptides specific for CD8 $^+$ T cells. The ICS did not show any difference in the frequencies of IFN- γ secreting CD4 $^+$ or CD8 $^+$ T cells between the mutant and any of the control viruses. The comparison of MVAwt and the MVA-BAC-85A showed similar vector-specific immune responses using CD8 $^+$ T cell-specific MVA peptides (E3 and F2(G) peptides). Moreover, the conventional MVA85A induced similar anti-vector and anti-rAg85A responses as the MVA-BAC-85A. Taking together, this supports our earlier observation [22] that inserting BAC DNA into the MVA genome did not alter the immune responses to the immunodominant vector epitopes E3 and F2(G) or to

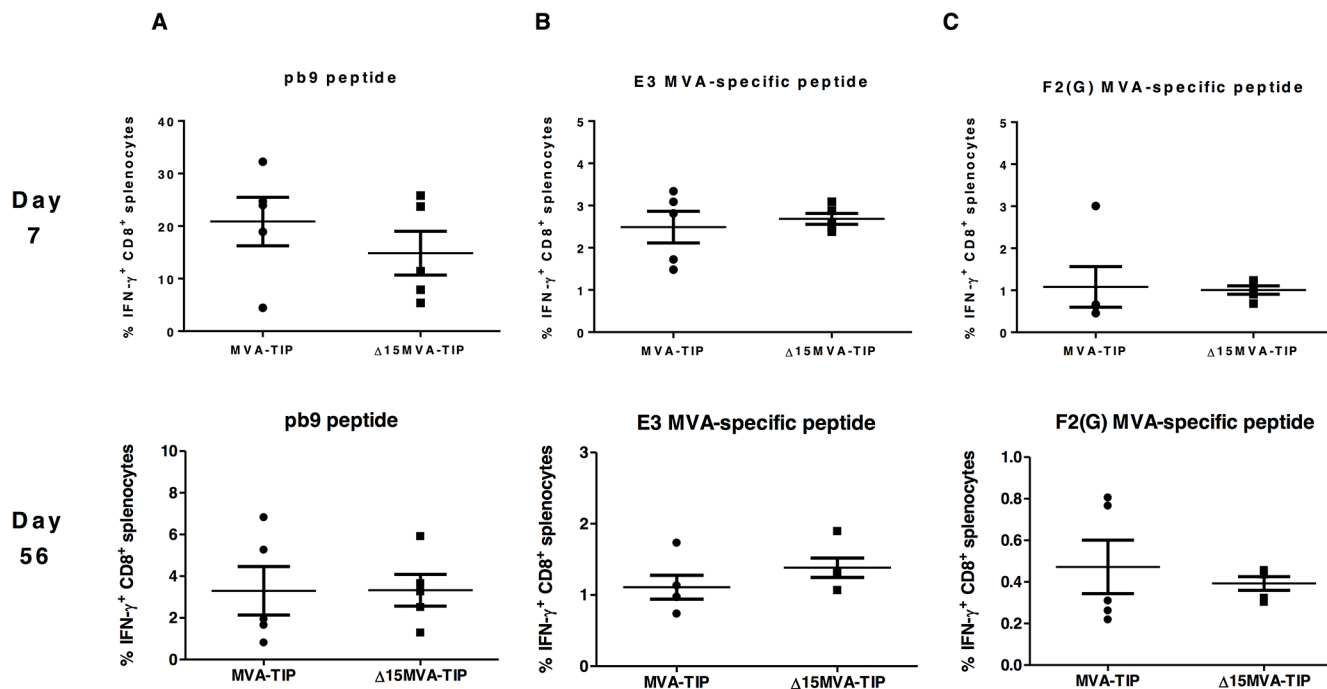


Fig 3. *In vivo* cellular immunogenicity of MVA deletion mutants, lacking fifteen genes, with TIP antigen, in a prime-boost regimen. Two groups of female BALB/c mice ($n = 10$) were immunized i.m. with 100 μ g of pSG-TIP DNA, then boosted with i.p. injection of MVAwt with TIP model antigen (MVA-TIP), or MVA deletion mutant (lacking 15 genes) Δ 15-MVA-TIP at the dose of 1×10^7 pfu/ml. **7 days** (top), or **56 days** (bottom) post-boost immunization, half of the mice were sacrificed and spleens collected for intracellular cytokine staining and flow cytometry to determine the percentage of IFN- γ -secreting CD8⁺ T splenocytes in response to *in vitro* re-stimulation with pb9 peptide (A), or with MVA vector-specific peptides (B and C). All values are presented after subtracting the values of unstimulated cells for every mouse (sample). The mean of each group with the SEM error bars are shown. Data is representative of two independent experiments. There was no statistical significant difference between the tested groups using Mann Whitney test.

doi:10.1371/journal.pone.0128626.g003

the recombinant antigens. Next, we tested the immune responses using two defined strong immune epitopes (named p15 and p11) specific to CD4⁺ or CD8⁺ T cells by ELISpot and observed a similar result to the ICS (Fig 4).

Discussion

Here we investigated the effect of deleting some immunomodulatory or unknown non-essential genes from MVA, in different combinations (Table 1), on the cellular immunogenicity. Deleting either few or many genes from MVA showed no difference in CD8⁺ T cells to the murine malaria epitope pb9 or to the vector-specific E3 and F2(G) epitopes. The use of TIP model antigen, which harbours the immunodominant pb9 epitope, enabled us to follow the immune responses for three months, but did not show any improvement at the long-term memory CD8⁺ T cell responses (for three months). Many previous studies showed that the single deletion of C6L [14], N2L [17] or C12 [18]; or the double deletion of A41L/B15R and C6L/K7R from MVA vectors enhanced the cellular immunogenicity in DNA-prime MVA-boost vaccination regimens. This regimen is also relevant to MVA clinical testing applications; MVA is used as a boosting agent in many vaccine clinical trials [26]. Thus, we applied this regimen priming with DNA, via i.m. route, and boosting with MVA, given i.p. Some of these genes, like A41L and B15R are deleted in the Δ 15-MVA-TIP mutant, and therefore this mutant would be expected to elicit stronger cellular immune responses, especially in this regimen as reported before. However, our result did not show any improved immunogenicity with the Δ 15-MVA-TIP in prime-only or prime-boost vaccinations. Garber *et al* (2012) reported that the deletion of four

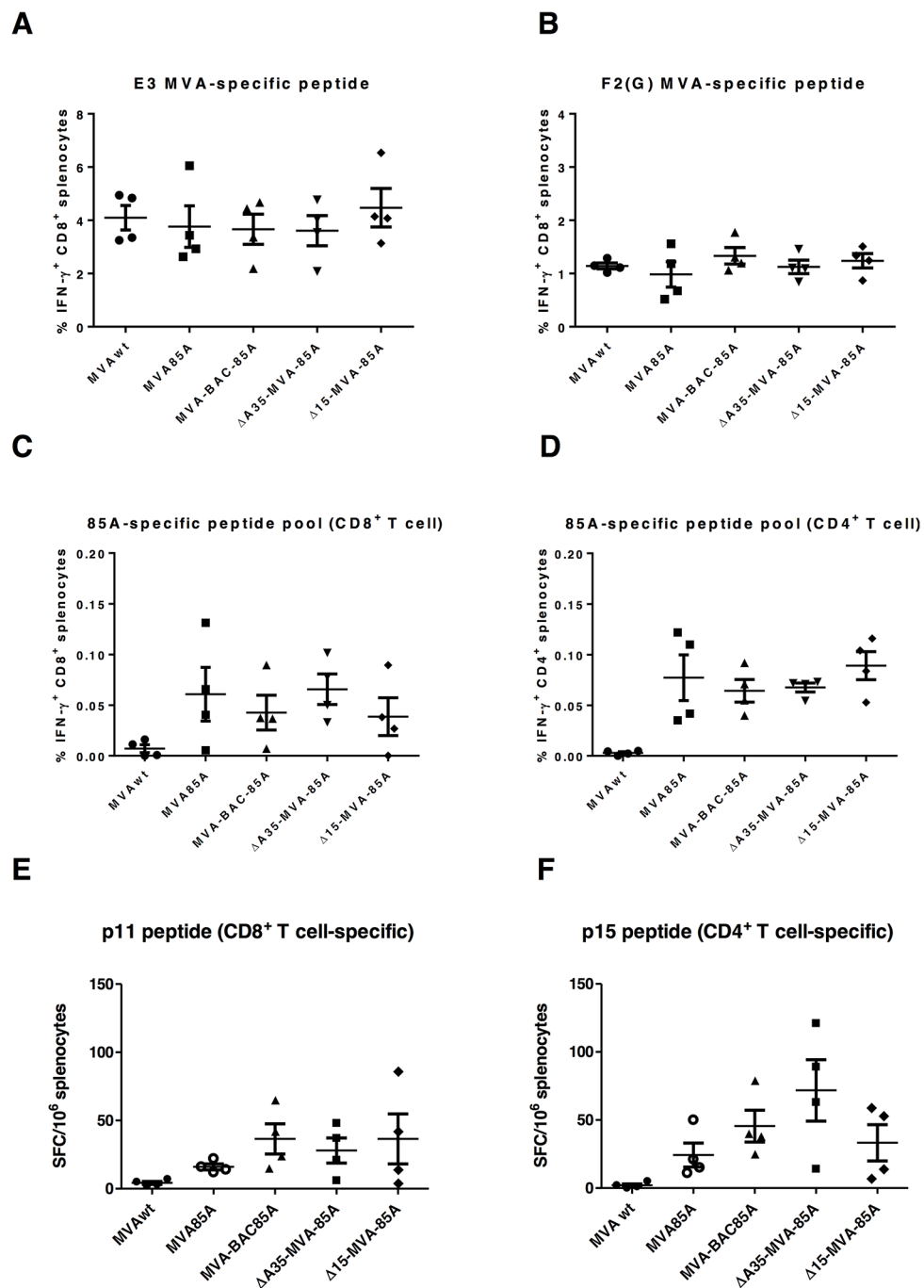


Fig 4. *In vivo* cellular immunogenicity of MVA deletion mutants, lacking fifteen genes, with 85A antigen. Four groups of female BALB/c mice ($n = 4$) were immunized (i.m.) with the respective MVAs at the dose of 2×10^6 pfu/ml. Seven days post immunization, intracellular cytokine staining and flow cytometry was performed to determine the percentage of splenic IFN- γ -secreting T cells in response to *in vitro* re-stimulation with MVA vector-specific peptide (**A** and **B**), with CD8⁺ T cell specific 85A peptide pool (**C**), or with CD4⁺ T cell specific 85A peptide pool (**D**). Two individual peptides were incubated for 18 hours to determine the CD8⁺ (**E**) or CD4⁺ (**F**) cell responses by ELISpot. These values are presented after subtracting the values of unstimulated cells for every mouse (sample). The mean of each group with the SEM error bars are shown. Data is representative of two independent experiments. There was no statistical significant difference in any of MVA mutant groups as compared to MVA85A or MVA-BAC85A groups, using Kruskal-Wallis test with Dunn's multiple comparisons. **MVAwt**: MVA wild type, **MVA85A**: MVA expressing TB 85A antigen, **MVA-BAC85A**: MVA, containing BAC DNA and expressing TB 85A antigen, and **Δ 15-MVA-85A**: MVA mutant, containing BAC DNA and expressing TB 85A antigen, with 15 gene deleted ([Table 1](#)).

doi:10.1371/journal.pone.0128626.g004

immunomodulatory genes enhanced MVA cellular immunogenicity in macaques, but the deletion of a fifth gene did not extend this improved immunogenicity [20]. Yet, when this fifth gene was deleted singly, the immunogenicity was enhanced in the same experimental setting [21]. It could be that the deletion of some genes could compensate for the absence of other deleted genes and the overall immunogenicity is not improved, but the unaltered immunogenicity with the large deletion mutant ($\Delta 15$ -MVA-TIP), which lacks *A41L* or *B15R*, was also noted when the *A41L* or *B15R* were singly deleted, as we previously published [22]. Moreover, these two genes were deleted in different cocktails of deleted gene-clusters in different mutants in the current study (Table 1) and none of these mutants showed any enhanced immune responses. This could rule out the possibility that the enhancement by some deletions could be rendered useless by other deleted genes of the same mutant. The deletion of *A41L* or *B15R*, or both of them was reported to enhance MVA cellular immune responses, but this enhancement was 2-fold at best [11–13]. Although there were some detailed technical or biological differences in each study concerning the deletion of *A41L* or *B15R*, or both, the inability to reproduce this improved immunogenicity could suggest that improving MVA immunogenic profiles could be very difficult to achieve through gene deletions, especially as MVA does not replicate *in vivo*, and its gene expression declines within 12 hours, and vanishes less than 48 hours post-inoculation *in vivo* [27, 28].

The concern over the use of non-natural model antigen like TIP should not apply to a “real” immunogenic antigen. The TB 85A is one of the most advanced candidates in clinical trials and showed strong CD4⁺ T and CD8⁺ T cell immunogenicity. Thus, we replaced TIP with 85A antigen in the large MVA deletion mutant (deriving $\Delta 15$ -MVA-85A), confirmed that no other changes had occurred in the genome by sequencing, then tested it in mice, measuring both CD4⁺ T and CD8⁺ T cell responses with peptide pools by ICS or two strong individual peptides (specific for CD4⁺ or CD8⁺ T cells) by ELISpot. Here, three control viruses were included; the MVA85A vaccine that is used for clinical testing against TB, the MVA-BAC-85A (which is similar to MVA85A, except it was derived via MVA-BAC recombineering), and the MVA wild type (MVA_{wt}), which is non-recombinant and non-mutant control. The results showed a continued lack of improved immunogenicity even with this natural immunogenic antigen. Although we were able to test CD4⁺ T cell responses, there was no significant difference in the magnitudes of 85A-specific CD4⁺ T cell between the mutant group and any of the control groups.

It could be speculated that the MVA-BAC system or deriving MVA in BHK-21 cell lines had some effect on these mutants, which could prevent them from enhancing the immune responses. The BHK-21 cell line was reported to allow for the replication of some MVA mutant lacking *E3L*, which is difficult to grow in CEFs [29]. This was because BHK-21 cell line expresses low concentration of IFN- α/β upon the infection of the E3-deleted MVA mutant, unlike CEFs that express high concentration of IFN- α/β , leading to abortive replication of the mutant [29]. The BHK-21 cells have also a low level of PKR (double-stranded RNA-dependent protein kinase), compared to the CEFs, which allowed for the growth of the E3-deleted MVA mutant [29]. The PKR is an important immune mediator in the NF κ B activation, so the growth of MVA mutants in BHK-21 cells may have less immune pressure. However, the proposed effect of MVA-BAC system or BHK-21 cells could be ruled out by comparing MVA85A and MVA-BAC-85A; the MVA85A was derived in the conventional method of constructing vaccinia virus recombinants and was propagated in CEFs whereas the MVA-BAC-85A was derived using MVA-BAC bacterial system and has undergone multiple rounds of MVA-BAC recombineering, then rescued using fowlpox virus and propagated in BHK-21 cell lines. Both viruses induced similar CD4⁺ and CD8⁺ T cell responses to the rAg-specific peptide pools by ICS or to the individual strong peptides measured by ELISpot as well as similar CD8⁺ T cell

responses to the vector-specific E3 and F2(G) peptides. Moreover, this was also supported by the result of MVA*wt* compared to the MVA-BAC-85A that showed similar responses to the vector specific E3 and F2(G) peptides. This MVA-BAC system was also shown to have no altering affect on the immunogenicity of MVA [22]. The MVA85A was included as a control to relate these modifications to the clinical testing as well, but we detected similar responses with MVA85A to the mutant and to the MVA-BAC-85A. In addition, the recombinant antigen-specific IFN- γ -secreting CD8⁺ T cells was the main readout as it would be more relevant for the improvement required for clinical testing of MVA-based vaccines.

The Δ C11-K3/B15-MVA lacks *C12L* and *C6L*, which were reported to enhance cellular immunogenicity when deleted from MVA [14, 18], did not show any difference in cellular immune responses. Again, the single deletion of *C12L* [22] or *C6L* (data not shown) failed to enhance the immune responses to TIP.

In conclusion, none of the derived rMVA mutants showed improved immunogenicity either to the MVA antigens or to the encoded TIP antigen. Focusing on the large deletion Δ 15-MVA-TIP mutant, neither prime only nor prime-boost regimens showed any improved CD8⁺ T cell responses. The memory responses at 28 or 84 (long-term) days post-MVA immunization showed no improved memory CD8⁺ T cell frequencies, except to one peptide (E3) at day 28. At day 56, in prime-boost regimen, this increase was observed but it was very marginal and insignificant. Even replacing TIP with TB 85A “real” antigen (Δ 15-MVA-85A) and testing CD8⁺ as well as CD4⁺ T cells magnitudes did not detect any differences compared to various control viruses. This study suggests that the previously reported immunogenicity increases in MVA deletion mutant, which were small increases in many cases (2-fold), may not be replicated with different antigens. The approach of gene deletion to improve MVA-vectored vaccines should be carefully assessed for every recombinant antigen, and epitope (peptide), and may not be taken as a generic approach for improving MVA immunogenicity.

Materials and Methods

Ethics Statement

All animal procedures were performed in accordance with the terms of the UK Animals (Scientific Procedures) Act (ASPA) for the project licenses 30/2414 or 30/2889 and were approved by the University of Oxford Animal Care and Ethical Review Committee. All mice were housed at least 7 days for settlement prior to any procedure in the University animal facility, Oxford, UK under Specific Pathogen Free (SPF) conditions.

Recombinant antigens

All rMVA mutants express a model antigen, called TIP [24] which is string of epitopes that encodes an H2-K^d restricted murine CD8⁺ T cell epitope SYIPSAEKI (pb9) from the *Plasmodium berghei* circumsporozoite protein [25]. TIP was inserted into MVA-BAC first, and then the MVA-BAC containing TIP was used as a template for making the described deletions (see Table 1). In the prime-boost regimen, the TIP was also cloned into pSG2 plasmid (pSG-TIP) and given i.m. at 100 μ g per mouse. In one rMVA mutant, Δ 15-MVA-TIP, the TIP was replaced with the *Mycobacterium tuberculosis* 85A antigen (TB 85A), deriving the Δ 15-MVA-85A. The 85A was also inserted into two other viruses to serve as controls; first, into a non-mutated MVA-BAC using MVA-BAC recombineering (virus named MVA-BAC-85A); second, into MVA wild type using the conventional recombinant vaccinia virus construction (named MVA85A, a current TB vaccine candidate in clinical trials [23]).

Construction of rMVA deletion mutants using MVA-BAC

Construction and generation of MVA-BAC and generation of rMVA deletion mutants using *GalK* recombineering [30] has been described previously [22]. To generate recombinant MVA (rMVA) viruses expressing TIP, a cassette was constructed using conventional PCR and restriction enzyme based cloning, comprising the TIP antigen, preceded by the early/late p7.5 viral promoter and the bacterial *GalK* resistance gene under a prokaryotic promoter. This was amplified with Phusion (Finnzymes) as a targeting DNA for recombineering by using long oligonucleotide primers (Eurofins MWG Operon) to add 50bp homology arms (matching two regions within the *TK* ORF) to the 5' and 3' ends. Primers were designed to insert the TIP-*GalK* cassette into the *TK* locus. These targeting constructs were used for MVA-BAC recombineering as previously described [22]. Next, MVA-BAC recombineering was performed to remove the *GalK* from MVA-BAC that contains TIP-*GalK*, leaving only the TIP into the *TK* locus. Therefore, *GalK* was then amplified with long oligonucleotide primers with 50 bp homology arms, matching the flanks of the targeted ORFs, deleting and replacing them with *GalK*. The colonies on *GalK*-positive plates were re-streaked three times before the recombineered MVA-BAC constructs are then confirmed by identity and purity PCR. The presence of the TIP was also confirmed by PCR and sequencing.

MVA-BAC rescue, and propagation and titration of rMVA mutants

The recombineered MVA-BACs were rescued to recombinant MVA in BHK cells using a fowlpox virus helper as previously described [22]. The *GalK* bacterial marker gene was only removed from mutants that required a multiple deletions such as the $\Delta 15$ -MVA-TIP, for the mutants with only one gene or one region of deleted genes, the *GalK* was not removed. BACs and derived viruses were checked for identity and purity by PCR and the sequences of the homology arms and transgenes were confirmed at both stages. BAC-derived rMVAs were plaque-picked three times to ensure purity, as a precautionary measure, since the bacterial colonies were re-streaked three times prior to rescue. The viruses were amplified in 1500cm² of BHK cell monolayers, purified over sucrose cushions and titred in BHK-21 cells according to standard practice, and purity and identity were again verified by PCR. Since MVA-BAC has a GFP marker gene under control of the Fowlpox virus p4B promoter [22], all the rMVAs expressed GFP, which was used for plaque picking and titration, in addition to the recombinant antigens.

Genome sequencing of the large rMVA deletion mutant, lacking fifteen genes

DNA samples of the $\Delta 15$ -MVA-85A from bacterial LB culture (for MVA-BAC DNA) or from BHK-21-infected cells (for viral DNA) were isolated using EndoFree Plasmid Maxi Kit (Qiagen) or standard phenol-chloroform DNA extraction, respectively. Samples were sent to the High-Throughput Genomics Group at the Wellcome Trust Centre for Human Genetics, University of Oxford, for MVA genome sequencing.

Peptides, spleen samples, and mouse immunogenicity

Female BALB/c mice aged 6 to 8 weeks (Harlan Laboratories, UK) were immunized intramuscularly (i.m.) in the tibialis muscles (50μL per mouse) or intradermally (i.d.) with a total of 10⁶ pfu of rMVA. For the heterologous prime boost regimen, mice were immunized with 100 μg of DNA, given i.m. (50μL per mouse) followed eight weeks later with 10⁷ pfu, given intraperitoneal (i.p.) of rMVA. Procedures were performed in accordance with the UK Animals (Scientific

Procedures) Act 1986 under granted project licenses 30/2414 or 30/2889. Splenocytes were harvested seven, or 56 days post-MVA immunization for analysis by IFN- γ ELISpot or intracellular cytokine staining (ICS) and flow cytometry as previously described [22, 31], using re-stimulation with 1 μ g/mL Pb9 peptide [25], 1 μ g/mL E3 and F2(G) peptides. or the long-term immunity assessment, samples were harvested at day 7, 28, and 84 post-MVA immunization. For the 85A responses, the re-stimulation was performed using CD4⁺ or CD8⁺ T cell-specific 85A peptide pools for ICS. Two 85A-specific individual peptides were used for ELISpot; CD8⁺ T cell-specific p11 peptide, EWYDQSGLSVVMPPVGGQSSF, and CD4⁺ T cell-specific p15 peptide, TFLTSELPGLWLQANRHVKPT.

Statistical analysis

GraphPad Prism (GraphPad software) was used for statistical analysis and to plot data.

Acknowledgments

The authors are grateful for the assistance of Matthew G. Cottingham (MGC, University of Oxford, UK). This work was funded in part by a Grand Challenges in Global Health grant to Prof. Adrian V. S. Hill, Jenner Institute, University of Oxford administered by the Foundation for the National Institutes of Health. AJS and MGC are Oxford Martin Fellows, SCG and AVS are Jenner Institute Investigators (<http://www.jenner.ac.uk>). NKA receives a scholarship fund from King Abdullah International Research Centre, Riyadh, Saudi Arabia (<http://www.kaimrc.med.sa>). We are grateful to the Jenner Institute's Vector Core Facility for technical assistance. We thank the High-Throughput Genomics Group at the Wellcome Trust Centre for Human Genetics (funded by Wellcome Trust grant reference 090532/Z/09/Z and MRC Hub grant G0900747 91070) for the generation of the sequencing data.

Author Contributions

Conceived and designed the experiments: NKA AJS AVS SCG. Performed the experiments: NKA AJS. Analyzed the data: NKA AJS. Contributed reagents/materials/analysis tools: NKA AJS AVS SCG. Wrote the paper: NKA.

References

1. Stickl H, Hochstein-Mintzel V, Mayr A, Huber HC, Schafer H, Holzner A. [MVA vaccination against smallpox: clinical tests with an attenuated live vaccinia virus strain (MVA) (author's transl)]. *Dtsch Med Wochenschr.* 1974; 99(47):2386–92. PMID: [4426258](#)
2. Carroll MW, Moss B. Host range and cytopathogenicity of the highly attenuated MVA strain of vaccinia virus: propagation and generation of recombinant viruses in a nonhuman mammalian cell line. *Virology.* 1997; 238(2):198–211. PMID: [9400593](#)
3. Mayr A, Stickl H, Muller HK, Danner K, Singer H. [The smallpox vaccination strain MVA: marker, genetic structure, experience gained with the parenteral vaccination and behavior in organisms with a debilitated defence mechanism (author's transl)]. *Zentralbl Bakteriol B.* 1978; 167(5–6):375–90. PMID: [749422](#)
4. Moorthy VS, McConkey S, Roberts M, Gothard P, Arulanantham N, Degano P, et al. Safety of DNA and modified vaccinia virus Ankara vaccines against liver-stage *P. falciparum* malaria in non-immune volunteers. *Vaccine.* 2003; 21(17–18):1995–2002. PMID: [12850357](#)
5. Sutter G, Staib C. Vaccinia vectors as candidate vaccines: the development of modified vaccinia virus Ankara for antigen delivery. *Current drug targets Infectious disorders.* 2003; 3(3):263–71. PMID: [14529359](#)
6. Draper SJ, Heeney JL. Viruses as vaccine vectors for infectious diseases and cancer. *Nature reviews Microbiology.* 2010; 8(1):62–73. doi: [10.1038/nrmicro2240](#) PMID: [19966816](#)
7. Gomez CE, Perdiguero B, Garcia-Arriaza J, Esteban M. Clinical applications of attenuated MVA poxvirus strain. *Expert review of vaccines.* 2013; 12(12):1395–416. doi: [10.1586/14760584.2013.845531](#) PMID: [24168097](#)

8. Antoine G, Schefflinger F, Dorner F, Falkner FG. The complete genomic sequence of the modified vaccinia Ankara strain: comparison with other orthopoxviruses. *Virology*. 1998; 244(2):365–96. PMID: [9601507](#)
9. Price PJ, Torres-Dominguez LE, Brandmuller C, Sutter G, Lehmann MH. Modified Vaccinia virus Ankara: innate immune activation and induction of cellular signalling. *Vaccine*. 2013; 31(39):4231–4. doi: [10.1016/j.vaccine.2013.03.017](#) PMID: [23523404](#)
10. Lousberg EL, Diener KR, Brown MP, Hayball JD. Innate immune recognition of poxviral vaccine vectors. *Expert review of vaccines*. 2011; 10(10):1435–49. doi: [10.1586/erv.11.121](#) PMID: [21988308](#)
11. Staib C, Kisling S, Erle V, Sutter G. Inactivation of the viral interleukin 1beta receptor improves CD8+ T-cell memory responses elicited upon immunization with modified vaccinia virus Ankara. *J Gen Virol*. 2005; 86(Pt 7):1997–2006. PMID: [15958679](#)
12. Clark RH, Kenyon JC, Bartlett NW, Tscharke DC, Smith GL. Deletion of gene A41L enhances vaccinia virus immunogenicity and vaccine efficacy. *J Gen Virol*. 2006; 87(Pt 1):29–38. PMID: [16361415](#)
13. Garcia-Arriaza J, Najera JL, Gomez CE, Sorzano CO, Esteban M. Immunogenic profiling in mice of a HIV/AIDS vaccine candidate (MVA-B) expressing four HIV-1 antigens and potentiation by specific gene deletions. *PloS one*. 2010; 5(8):e12395. doi: [10.1371/journal.pone.0012395](#) PMID: [20811493](#)
14. Garcia-Arriaza J, Najera JL, Gomez CE, Tewabe N, Sorzano CO, Calandra T, et al. A candidate HIV/AIDS vaccine (MVA-B) lacking vaccinia virus gene C6L enhances memory HIV-1-specific T-cell responses. *PloS one*. 2011; 6(8):e24244. doi: [10.1371/journal.pone.0024244](#) PMID: [21909386](#)
15. Garcia-Arriaza J, Arnaez P, Gomez CE, Sorzano CO, Esteban M. Improving Adaptive and Memory Immune Responses of an HIV/AIDS Vaccine Candidate MVA-B by Deletion of Vaccinia Virus Genes (C6L and K7R) Blocking Interferon Signaling Pathways. *PloS one*. 2013; 8(6):e66894. PMID: [23826170](#)
16. Perdiguero B, Gomez CE, Najera JL, Sorzano CO, Delaloye J, Gonzalez-Sanz R, et al. Deletion of the viral anti-apoptotic gene F1L in the HIV/AIDS vaccine candidate MVA-C enhances immune responses against HIV-1 antigens. *PloS one*. 2012; 7(10):e48524. doi: [10.1371/journal.pone.0048524](#) PMID: [23119046](#)
17. Garcia-Arriaza J, Gomez CE, Sorzano CO, Esteban M. Deletion of the vaccinia virus N2L gene encoding an inhibitor of IRF3 improves the immunogenicity of modified vaccinia virus Ankara expressing HIV-1 antigens. *Journal of virology*. 2014; 88(6):3392–410. doi: [10.1128/JVI.02723-13](#) PMID: [24390336](#)
18. Falivene J, Del Medico Zajac MP, Pascutti MF, Rodriguez AM, Maeto C, Perdiguero B, et al. Improving the MVA vaccine potential by deleting the viral gene coding for the IL-18 binding protein. *PloS one*. 2012; 7(2):e32220. doi: [10.1371/journal.pone.0032220](#) PMID: [22384183](#)
19. Rehm KE, Roper RL. Deletion of the A35 gene from Modified Vaccinia Virus Ankara increases immunogenicity and isotype switching. *Vaccine*. 2011; 29(17):3276–83. doi: [10.1016/j.vaccine.2011.02.023](#) PMID: [21352940](#)
20. Garber DA, O'Mara LA, Gangadhara S, McQuoid M, Zhang X, Zheng R, et al. Deletion of specific immune-modulatory genes from modified vaccinia virus Ankara-based HIV vaccines engenders improved immunogenicity in rhesus macaques. *Journal of virology*. 2012; 86(23):12605–15. doi: [10.1128/JVI.00246-12](#) PMID: [22973033](#)
21. Garber DA, O'Mara LA, Zhao J, Gangadhara S, An I, Feinberg MB. Expanding the repertoire of Modified Vaccinia Ankara-based vaccine vectors via genetic complementation strategies. *PloS one*. 2009; 4(5):e5445. doi: [10.1371/journal.pone.0005445](#) PMID: [19421328](#)
22. Cottingham MG, Andersen RF, Spencer AJ, Saurya S, Furze J, Hill AV, et al. Recombination-mediated genetic engineering of a bacterial artificial chromosome clone of modified vaccinia virus Ankara (MVA). *PloS one*. 2008; 3(2):e1638. doi: [10.1371/journal.pone.0001638](#) PMID: [18286194](#)
23. Tameris MD, Hatherill M, Landry BS, Scriba TJ, Snowden MA, Lockhart S, et al. Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: a randomised, placebo-controlled phase 2b trial. *Lancet*. 2013; 381(9871):1021–8. PMID: [23391465](#)
24. Alcock R, Cottingham MG, Rollier CS, Furze J, De Costa SD, Hanlon M, et al. Long-term thermostabilization of live poxviral and adenoviral vaccine vectors at supraphysiological temperatures in carbohydrate glass. *Science translational medicine*. 2010; 2(19):19ra2.
25. Romero P, Maryanski JL, Cordey AS, Corradin G, Nussenzweig RS, Zavala F. Isolation and characterization of protective cytolytic T cells in a rodent malaria model system. *Immunology letters*. 1990; 25(1–3):27–31. PMID: [2249878](#)
26. Gilbert SC, Moorthy VS, Andrews L, Pathan AA, McConkey SJ, Vuola JM, et al. Synergistic DNA-MVA prime-boost vaccination regimes for malaria and tuberculosis. *Vaccine*. 2006; 24(21):4554–61. PMID: [16150517](#)

27. Ramirez JC, Gherardi MM, Esteban M. Biology of attenuated modified vaccinia virus Ankara recombinant vector in mice: virus fate and activation of B- and T-cell immune responses in comparison with the Western Reserve strain and advantages as a vaccine. *Journal of virology*. 2000; 74(2):923–33. PMID: [10623755](#)
28. Geiben-Lynn R, Greenland JR, Frimpong-Boateng K, Letvin NL. Kinetics of recombinant adenovirus type 5, vaccinia virus, modified vaccinia ankara virus, and DNA antigen expression in vivo and the induction of memory T-lymphocyte responses. *Clinical and vaccine immunology: CVI*. 2008; 15(4):691–6. doi: [10.1128/CVI.00418-07](#) PMID: [18272665](#)
29. Hornemann S, Harlin O, Staib C, Kisling S, Erfle V, Kaspers B, et al. Replication of modified vaccinia virus Ankara in primary chicken embryo fibroblasts requires expression of the interferon resistance gene E3L. *Journal of virology*. 2003; 77(15):8394–407. PMID: [12857909](#)
30. Warming S, Costantino N, Court DL, Jenkins NA, Copeland NG. Simple and highly efficient BAC recombineering using galK selection. *Nucleic acids research*. 2005; 33(4):e36. PMID: [15731329](#)
31. Sridhar S, Reyes-Sandoval A, Draper SJ, Moore AC, Gilbert SC, Gao GP, et al. Single-dose protection against *Plasmodium berghei* by a simian adenovirus vector using a human cytomegalovirus promoter containing intron A. *Journal of virology*. 2008; 82(8):3822–33. doi: [10.1128/JVI.02568-07](#) PMID: [18256155](#)
32. Buller RM, Chakrabarti S, Moss B, Fredrickson T. Cell proliferative response to vaccinia virus is mediated by VGF. *Virology*. 1988; 164(1):182–92. PMID: [3363864](#)
33. Kluczyk A, Siemion IZ, Szewczuk Z, Wieczorek Z. The immunosuppressive activity of peptide fragments of vaccinia virus C10L protein and a hypothesis on the role of this protein in the viral invasion. *Peptides*. 2002; 23(5):823–34. PMID: [12084512](#)
34. Reading PC, Smith GL. Vaccinia virus interleukin-18-binding protein promotes virulence by reducing gamma interferon production and natural killer and T-cell activity. *Journal of virology*. 2003; 77(18):9960–8. PMID: [12941906](#)
35. Ahn BY, Jones EV, Moss B. Identification of the vaccinia virus gene encoding an 18-kilodalton subunit of RNA polymerase and demonstration of a 5' poly(A) leader on its early transcript. *Journal of virology*. 1990; 64(6):3019–24. PMID: [2335825](#)
36. Oguiura N, Spehner D, Drillien R. Detection of a protein encoded by the vaccinia virus C7L open reading frame and study of its effect on virus multiplication in different cell lines. *J Gen Virol*. 1993; 74 (Pt 7):1409–13. PMID: [8336123](#)
37. Najera JL, Gomez CE, Domingo-Gil E, Gherardi MM, Esteban M. Cellular and biochemical differences between two attenuated poxvirus vaccine candidates (MVA and NYVAC) and role of the C7L gene. *Journal of virology*. 2006; 80(12):6033–47. PMID: [16731942](#)
38. Unterholzner L, Sumner RP, Baran M, Ren H, Mansur DS, Bourke NM, et al. Vaccinia virus protein C6 is a virulence factor that binds TBK-1 adaptor proteins and inhibits activation of IRF3 and IRF7. *PLoS pathogens*. 2011; 7(9):e1002247. doi: [10.1371/journal.ppat.1002247](#) PMID: [21931555](#)
39. Cooray S, Bahar MW, Abrescia NG, McVey CE, Bartlett NW, Chen RA, et al. Functional and structural studies of the vaccinia virus virulence factor N1 reveal a Bcl-2-like anti-apoptotic protein. *J Gen Virol*. 2007; 88(Pt 6):1656–66. PMID: [17485524](#)
40. Ferguson BJ, Benfield CT, Ren H, Lee VH, Frazer GL, Strnadova P, et al. Vaccinia virus protein N2 is a nuclear IRF3 inhibitor that promotes virulence. *J Gen Virol*. 2013; 94(Pt 9):2070–81. doi: [10.1099/vir.0.054114-0](#) PMID: [23761407](#)
41. Staib C, Drexler I, Ohlmann M, Wintersperger S, Erfle V, Sutter G. Transient host range selection for genetic engineering of modified vaccinia virus Ankara. *BioTechniques*. 2000; 28(6):1137–42, 44–6, 48. PMID: [10868279](#)
42. Law KM, Smith GL. A vaccinia serine protease inhibitor which prevents virus-induced cell fusion. *J Gen Virol*. 1992; 73 (Pt 3):549–57. PMID: [1545218](#)
43. Rice AD, Turner PC, Embury JE, Moldawer LL, Baker HV, Moyer RW. Roles of vaccinia virus genes E3L and K3L and host genes PKR and RNase L during intratracheal infection of C57BL/6 mice. *Journal of virology*. 2011; 85(1):550–67. doi: [10.1128/JVI.00254-10](#) PMID: [20943971](#)
44. Alejo A, Ruiz-Arguello MB, Ho Y, Smith VP, Saraiva M, Alcamí A. A chemokine-binding domain in the tumor necrosis factor receptor from variola (smallpox) virus. *Proc Natl Acad Sci U S A*. 2006; 103(15):5995–6000. PMID: [16581912](#)
45. Symons JA, Tschärke DC, Price N, Smith GL. A study of the vaccinia virus interferon-gamma receptor and its contribution to virus virulence. *J Gen Virol*. 2002; 83(Pt 8):1953–64. PMID: [12124459](#)
46. Price N, Tschärke DC, Smith GL. The vaccinia virus B9R protein is a 6 kDa intracellular protein that is non-essential for virus replication and virulence. *J Gen Virol*. 2002; 83(Pt 4):873–8. PMID: [11907337](#)

47. Banham AH, Smith GL. Characterization of vaccinia virus gene B12R. *J Gen Virol.* 1993; 74 (Pt 12):2807–12. PMID: [8277291](#)
48. Machesky LM, Cole NB, Moss B, Pollard TD. Vaccinia virus expresses a novel profilin with a higher affinity for polyphosphoinositides than actin. *Biochemistry.* 1994; 33(35):10815–24. PMID: [8075084](#)
49. Sood CL, Moss B. Vaccinia virus A43R gene encodes an orthopoxvirus-specific late non-virion type-1 membrane protein that is dispensable for replication but enhances intradermal lesion formation. *Virology.* 2010; 396(1):160–8. doi: [10.1016/j.virol.2009.10.025](#) PMID: [19900687](#)
50. Duncan SA, Smith GL. Vaccinia virus gene Salf5R is non-essential for virus replication in vitro and in vivo. *J Gen Virol.* 1992; 73 (Pt 5):1235–42. PMID: [1588324](#)
51. Reading PC, Moore JB, Smith GL. Steroid hormone synthesis by vaccinia virus suppresses the inflammatory response to infection. *The Journal of experimental medicine.* 2003; 197(10):1269–78. PMID: [12756265](#)
52. Almazan F, Tschärke DC, Smith GL. The vaccinia virus superoxide dismutase-like protein (A45R) is a virion component that is nonessential for virus replication. *Journal of virology.* 2001; 75(15):7018–29. PMID: [11435582](#)
53. Stack J, Haga IR, Schroder M, Bartlett NW, Maloney G, Reading PC, et al. Vaccinia virus protein A46R targets multiple Toll-like-interleukin-1 receptor adaptors and contributes to virulence. *The Journal of experimental medicine.* 2005; 201(6):1007–18. PMID: [15767367](#)