



Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.

The attenuation of vaccinia Tian Tan strain by the removal of the viral M1L-K2L genes

Weijun Zhu^{a,1}, Qing Fang^{a,1}, Ke Zhuang^a, Haibo Wang^a, Wenbo Yu^a,
Jingying Zhou^a, Li Liu^a, Po Tien^a, Linqi Zhang^{a,b,c}, Zhiwei Chen^{a,b,*}

^a Modern Virology Research Center and AIDS Center, State Key Laboratory of Virology, College of Life Sciences, Wuhan University, Hubei 430072, PR China

^b Aaron Diamond AIDS Research Center, The Rockefeller University, New York, NY 10016, USA

^c AIDS Research Center, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100730, PR China

Received 21 July 2006; received in revised form 12 March 2007; accepted 14 March 2007

Available online 24 April 2007

Abstract

To generate a safe vaccinia Tian Tan (VTT)-based vaccine vector, it is necessary to develop a method to attenuate the virus. A modified VTT (MVTT_{2-GFP}) was constructed by replacing the viral M1L-K2L genes with a GFP gene. In comparison to the parental VTT, MVTT_{2-GFP} lost its replication capacity in rabbit RK13 and human HeLa cell lines. The life cycle of viral replication was blocked at different stages in these two cell lines as determined by electron microscope examination. MVTT_{2-GFP} was less virulent than VTT for 100-fold by measuring mouse body weight loss after intranasal viral inoculation and for 340-fold by determining the intracranial LD₅₀ value in mice. The foreign GFP gene was stable genetically after 10 rounds of passage in Vero cells. Importantly, MVTT_{2-GFP} elicited both humoral and cell-mediated immune responses to the GFP gene in mice. With two intramuscular inoculations of 10⁵ PFU virus, the anti-GFP antibody reciprocal endpoint titer reached over 700 as determined by an ELISA. The number of IFN- γ secreting T cells reached over 350 SFU per million splenocytes against a CD8+ T cell-specific epitope of GFP. Collectively, the removal of the M1L-K2L genes is a useful method to generate an attenuated vaccinia Tian Tan vaccine vector.

© 2007 Elsevier B.V. All rights reserved.

Keywords: Vaccinia; Vaccine; Viral vector; Vaccinia Tian Tan; MVTT

1. Introduction

After the declaration of the worldwide eradication of smallpox in 1980, vaccinia virus has been studied for use as a live viral vector for other infectious diseases or cancer therapy (Moss, 1996; Mwau et al., 2004; Paoletti, 1996). In the People's Repub-

lic of China, the vaccinia Tian Tan (VTT) strain was used historically as a smallpox vaccine for millions of people, yet its potential as a vaccine vector has not been explored carefully. The full-length sequence of the VTT genome was determined by Jin et al. (1997). Based on sequence comparison, VTT displays genetic features distinct from other vaccinia viruses including the vaccinia western reserve (WR) strain (Hou et al., 1985; Jin et al., 1997; Tsao et al., 1986). In particular, unique deletions or insertions, which affect multiple genes, were identified in the restriction enzyme *HindIII*-C, B and A fragments. These data infer the plausibly distinct biological properties of VTT (Jin et al., 1997). Recently, the biological properties of VTT were characterized in terms of its host cell range and growth properties *in vitro* and virulence *in vivo* (Fang et al., 2005). Although VTT is significantly less virulent than the vaccinia WR strain, it remains lethal in mice after intracranial inoculation and causes significant body weight loss after intranasal inoculation (Fang et al., 2005). These properties have limited its use as a vaccine

Abbreviations: VTT, vaccinia Tian Tan; MVTT, modified vaccinia Tian Tan; MVA, modified vaccinia Ankara; GFP, green fluorescent protein; AIDS, acquired immunodeficiency syndrome; HIV-1, human immunodeficiency virus type one; SIV, simian immunodeficiency virus; SHIV, simian/human immunodeficiency chimeric virus; CTL, cytotoxic T lymphocytes; MOI, multiplicity of infection; PFU, plaque forming unit; p.i., post-infection; EM, electron microscope

* Corresponding author at: Aaron Diamond AIDS Research Center, 455 First Avenue, New York, NY 10016, USA. Tel.: +1 212 448 5031; fax: +1 212 725 1126.

E-mail address: zchen@adarc.org (Z. Chen).

¹ These authors contributed equally to this work.

vector for human use, especially for immune-suppressed individuals. Therefore, further attenuation of VTT is necessary for the development of a useful vaccine vector.

Various viral vector systems have been evaluated for vaccine development (Moss et al., 1996; Santra et al., 2005; Tartaglia et al., 1992). The poxvirus vector is the live recombinant vector which has been studied most intensively. The widely studied vaccinia-based vector is probably the modified vaccinia virus Ankara (MVA). This vector is safe in humans, even in some immunocompromised individuals (Cosma et al., 2003). Moreover, MVA-based vaccines are effective for inducing protective responses against different viruses, such as severe acute respiratory syndrome coronavirus, influenza and respiratory syncytial virus (Bisht et al., 2004; Chen et al., 2005; De Waal et al., 2004; Degano et al., 1999; Olszewska et al., 2004; Sutter et al., 1994; Wyatt et al., 1999). MVA-based vaccines are also effective in delaying the development of AIDS and the progression of the disease in rhesus monkeys infected with simian immunodeficiency virus (SIV) or simian/human immunodeficiency virus (SHIV) (Barouch et al., 2001). However, the immunogenicity profile of MVA as a vaccine vector for HIV-1 is unsatisfactory in humans. A recent phase one trial indicated that an MVA-based HIV-1 vaccine is not very immunogenic in humans (Goonetilleke et al., 2006). It is, therefore, desirable to explore the potential of other vaccinia-based vector systems for stimulating stronger host immune responses in humans.

In this study, a method to generate a modified VTT (MVTT_{2-GFP}) is described. This method involves the genetic modification of the parental VTT genome by deleting the M1L-K2L genes including one host range gene, K1L. The rationale for choosing these genes for deletion corresponds to the lost genes for the deletion II region of MVA genome (Antoine et al., 1998; Meyer et al., 1991). Before this study, it was unknown to what extent a vaccinia virus could be attenuated just by removing the M1L-K2L genes. As the deletion II region of MVA serves as an ideal insertion site for foreign genes, the effectiveness of the corresponding region in the VTT genome for the expression of a foreign gene was also unknown. For these reasons, the phenotypic changes of MVTT_{2-GFP} in comparison to the parental VTT both *in vitro* and *in vivo*, and the potential use of MVTT_{2-GFP} as a live viral vector for vaccine development were examined in this study.

2. Materials and methods

2.1. Cell lines and viruses

Thirteen cell lines of various origins were maintained under growth conditions suggested by the American Type Culture Collection (ATCC, Rockville, MD). These cell lines include COS-7, WISH, BHK-21, MRC-5, CHO-K1, HeLa, 293T, RK(15), MDCK, RK13, C6, Vero and NIH/3T3. Primary chicken embryo fibroblasts (CEF) were prepared from 10-day-old embryos. The parental vaccinia Tian Tan 761 strain was obtained from the Institute of Virology at the Chinese Center for Disease Control and Prevention. This VTT 761 strain was derived

originally from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (NICPBP). Virus stocks were propagated in Vero cells and then purified by centrifugation through a 36% sucrose cushion. The virus stocks were titrated by a plaque forming assay using crystal violet staining.

2.2. Construction of shuttle vector and MVTT_{2-GFP}

The shuttle vector was constructed by inserting two genome fragments flanking the deletion region of VTT. The GFP gene was placed under a synthetic early/late promoter P_{syn} (Chakrabarti et al., 1997; Wyatt et al., 1996). The MVTT_{2-GFP} virus was made by a homologous recombination method as described previously (Sutter and Moss, 1992; Sutter et al., 1994; Wyatt et al., 1996). The plaques that express GFP were selected and purified subsequently through six rounds of plaque purification under agarose. The insert in the recombinant virus was determined by a nested PCR using primer pairs flanking the deletion region. PCR primers used for this test were: 5'-GTTTATACAATCCATGCTACTACCTTCGGG-3' (sense) and 5'-GAATTTCCATTACATCAGGCAGCCACATTGGAAG-3' (antisense) for the first round reaction and 5'-GGTTCATTGTTATCCATTGCAGAGGACG-3' (sense) and 5'-GTAAAGATTTTGCTATTTCAGTGGACTGGATG-3' (antisense) for the second round of reaction. The amplification cycles are 95 °C for 2 min followed by 35 cycles of 94 °C for 20 s, 52 °C for 45 s and 72 °C for 3 min plus the last extension of 72 °C for 8 min. Amplified PCR products were purified using a QIAquick PCR purification kit (QIAGEN, Valencia, CA, USA) and were subjected to DNA sequencing directly by an automated ABI 377 DNA sequencer (Applied Biosystems Inc., Foster City, CA, USA). For comparison, a recombinant VTT_{GFP} was generated by placing GFP gene in a location upstream of the hemagglutinin gene without artificial interruption of the M1L-K2L genes.

2.3. Host cell range, growth property and virulence of MVTT_{2-GFP}

Assays for the determination of viral host cell range, growth property *in vitro* and virulence of MVTT_{2-GFP} *in vivo* have been described previously (Carroll and Moss, 1997; Fang et al., 2005).

2.4. Electron microscopic (EM) testing

Confluent cell monolayers were infected with 5 MOI of MVTT_{2-GFP} or VTT. The virus was allowed to attach to the cells for 90 min at 37 °C. The cells were then washed with medium three times and incubated at 37 °C for an additional 16 h. The infected cells were then trypsinized to detach them from the culture plates and washed with PBS twice. The cell pellets were fixed in 2.5% glutaraldehyde and processed for examination using a transmission electron microscopy by a routine technique described previously (Wolffe et al., 1996).

2.5. Stability of the GFP gene in MVTT_{2-GFP}

After a six-round purification of the GFP positive plaques, ten additional rounds of passage of the virus were carried out. After the last passage, Vero cells were infected subsequently with MVTT_{2-GFP} at an MOI of 0.01. Forty-eight hours after infection, the percentages of GFP and vaccinia antigen double positive plaques were counted. The method for the immunostaining of vaccinia specific antigens has been described previously (Carroll and Moss, 1997).

2.6. In vivo immunogenicity of MVTT_{2-GFP}

BALB/c mice were immunized with MVTT_{2-GFP}. Groups of three 6–8-week-old female BALB/c mice were immunized intramuscularly twice with 10⁵ PFU of MVTT_{2-GFP} or the parental type VTT in 100 µl of PBS at weeks 0 and 3. Serum samples were collected at weeks 0, 3 and 5 for anti-GFP antibody testing (Gambotto et al., 2000; Hsu et al., 2005). The anti-GFP antibody endpoint titer was determined as the reciprocal highest dilution of samples producing at least two-fold greater than the value corresponding to the optical density readout of the control serum sample at the same dilution. The coating antigen was recombinant His-tagged EGFP that was purified from *E. coli* by nickel column chromatography. At week 5, the mice were killed and splenocytes were isolated from the mice for measuring the cell-mediated immune response by an ELISpot assay Kit (U-Cytech, The Netherlands). A CD8⁺-specific epitope HYLSTQSAL was used as the pulsing antigen (Gambotto et al., 2000). The mice received PBS served as the negative controls. *Staphylococcus aureus* enterotoxin B (SEB) was used as a positive control. Spots were counted by using an ELISpot reader (BIOREADER 4000, Bio-sys Inc., Germany).

2.7. Neutralization assay

Serial two-fold dilutions of heat-inactivated mouse serum were incubated with 100 PFU of the parental VTT at 37 °C for 1 h. The mixed solution was then inoculated onto Vero cells in 24-well plates. The cells were over-layered by agarose after 90 min of viral absorption. After 48 h of incubation at 37 °C, the viral plaques were visualized by crystal violet staining and counted. Antibody titers were defined as the reciprocal of serum dilution that reduced viral plaques by 50%. Serum samples from

pre-immunized or mock-immunized animals were included as background controls.

3. Results

3.1. Construction of MVTT_{2-GFP}

Using a homologous recombination method, a GFP gene was introduced into the VTT genome to generate the MVTT_{2-GFP}. The introduction of the GFP gene replaced a fragment of viral genome from position 22,372 to 25,187 bases of VTT (GenBank accession number AF095689). This replacement resulted in the deletion of viral M1L-K2L genes (Tan-TM1L, Tan-TM2L, Tan-TK1L and Tan-TK2L) (Table 1) (Drillien et al., 1981). This replacement was successful as the plaque-purified GFP positive virus was tested by a specific PCR, immunochemical staining and subsequent sequence analysis (data not shown). After a series of plaque purifications, a pure viral stock of MVTT_{2-GFP} was generated as determined by the co-expression of GFP and vaccinia specific antigens (data not shown).

3.2. Reduced replication capacity of MVTT_{2-GFP}

To determine the effect of these deleted VTT genes on the viral biological properties, the growth kinetics of MVTT_{2-GFP} were quantitatively characterized using a previously described method (Carroll and Moss, 1997). Thirteen mammalian cell lines of various host origin and primary chicken embryo fibroblasts (CEF) were infected with MVTT_{2-GFP} at an MOI of 0.05. These cell lines included COS-7, WISH, BHK-21, MRC-5, CHO-K1, HeLa, 293T, RK(15), MDCK, RK13, C6, Vero and NIH/3T3. Since these cells were derived from diverse host and tissue origins, this experiment determined the breadth of the host cell range of MVTT_{2-GFP}. In comparison to the parental VTT, the replication property of MVTT_{2-GFP} did not change significantly in cell lines including COS-7, Vero, WISH, BHK-21, MRC-5, CHO-K1, NIH/3T3 and CEF. For example, MVTT_{2-GFP} replicates well in Vero cells (Fig. 1). However, the replication level of MVTT_{2-GFP} dropped more than 6–10-fold in cell lines including MDCK, C6, RK13, PK(15), 293T and HeLa (Fig. 1 and Table 2). In particular, MVTT_{2-GFP} did not seem to replicate in RK13 and HeLa cells overtime. These results indicate that the loss of viral M1L-K2L genes likely has a significant impact on the growth capacity of the virus in certain cell lines *in vitro*.

Table 1
Genes deleted in the genome of MVTT_{2-GFP}

Gene	Name in PBRC ^a	Expression	Functions	References
M1L	VV-Tan-TM1L	Early	Ankyrin-like protein	Shchelkunov et al. (1993)
M2L	VV-Tan-TM2L	Early	Unknown	Jing et al. (2005)
K1L	VV-Tan-TK1 L	Early	Ankyrin/host range	Chung et al. (1997), Gillard et al. (1986), Perkus et al. (1990), Ramsey-Ewing and Moss (1996) and Shisler and Jin (2004)
K2L	VV-Tan-TK2L	Intermediate	Serine proteases inhibitor (SPI), prevent cell fusion	Gambotto et al. (2000), Hsu et al. (2005), Law and Smith (1992), Turner and Moyer (1995) and Zhou et al. (1992)

^a PBRC: Poxvirus Bioinformatics Resource Center.

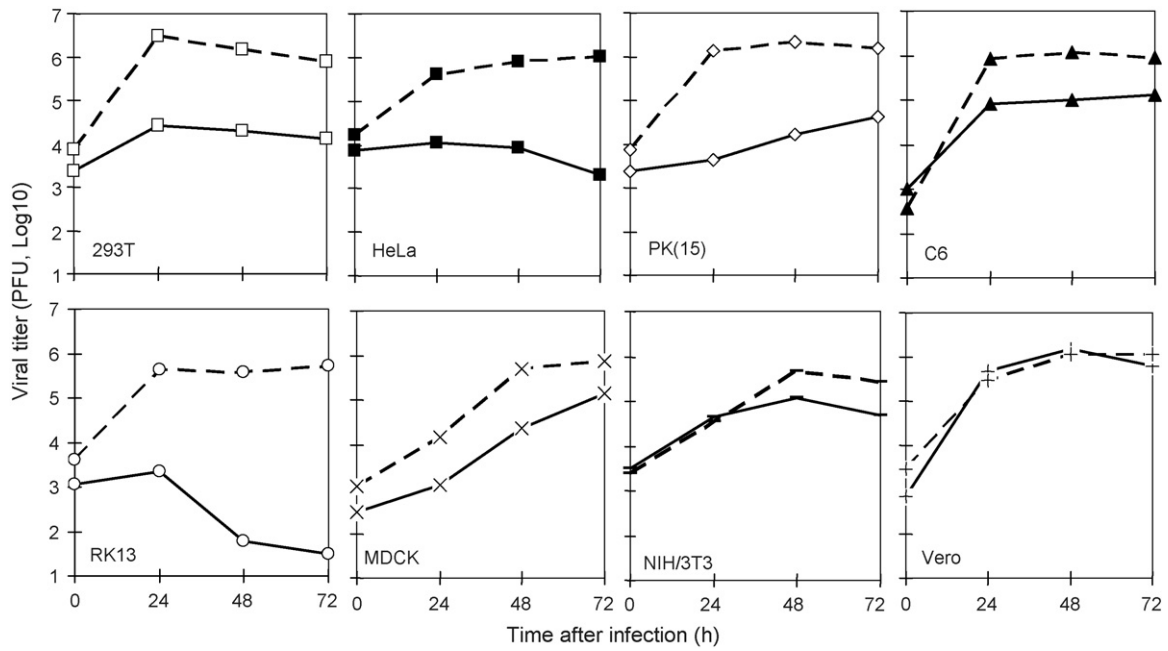


Fig. 1. The replication of MVTT_{2-GFP} (solid line) and VTT (dashed line) in eight cell lines tested. Cells were infected with an MOI of 0.05. The viral replication was determined by measuring the viral titer after viral absorption, and 24, 48, and 72 h p.i. Both culture medium and cells were harvested for testing in these experiments. Symbols are defined as follows: (□) 293T, (■) HeLa, (◇) PK(15), (○) RK13, (×) MDCK, (▲) C6, (–) NIH/3T3, and (+) Vero. This experiment was repeated twice with similar results obtained.

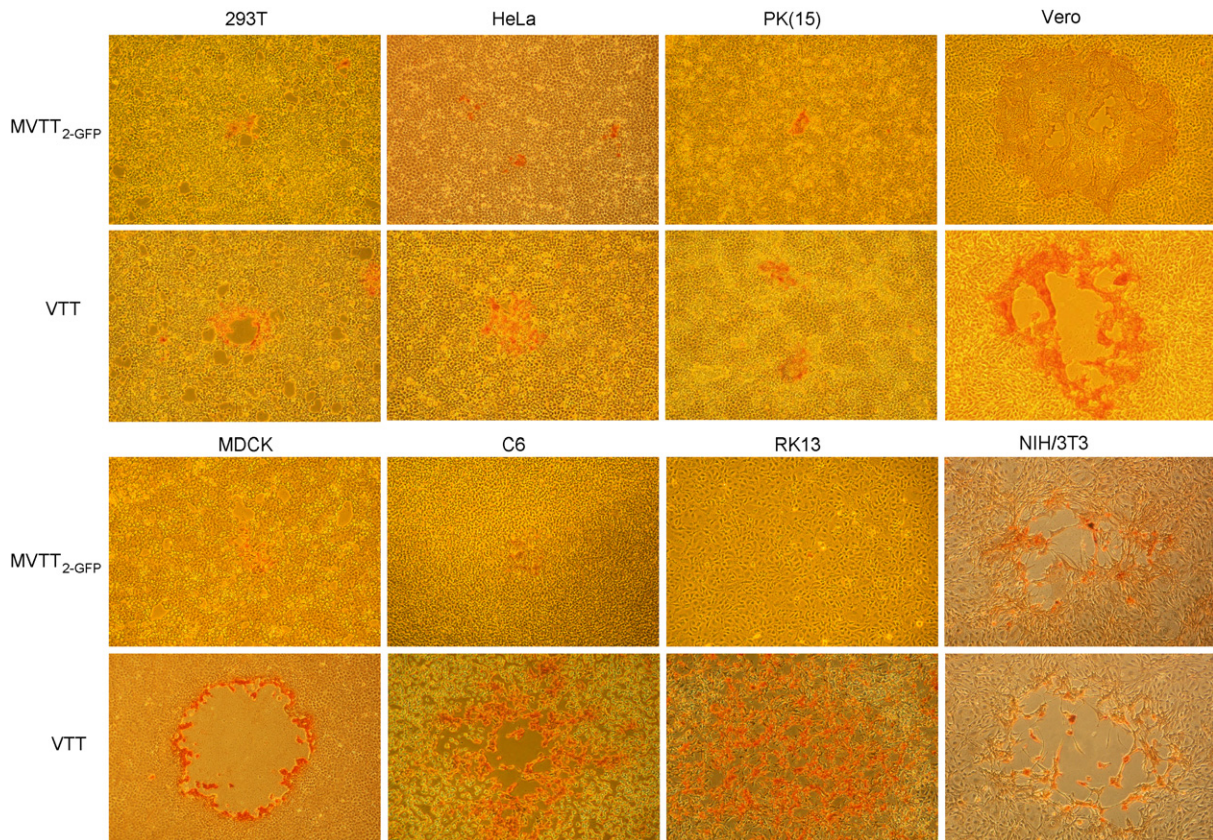


Fig. 2. Infection and cell-to-cell spread of MVTT_{2-GFP} or VTT in eight cell types. The cells were infected with an MOI of 0.01, then fixed and immunostained with a specific anti-vaccinia virus serum at 48 h p.i. The panels show representative fields at an approximately 100× magnification. This experiment was repeated three times with similar results obtained.

Table 2
Replication and spread of viruses in various cell types

Cell type	ATCC code	Species	Organ	Morphology	Virus spread ^a		Virus replication ^b (MVTT ₂ -GFP)	
					MVTT ₂ -GFP	VTT		
He La	CCL-2	Human	Cervix	Epithelial	++	+++	0.004	NP
293T	CRL-11268	Human	Kidney	Epithelial	++	+++	2.5	SP
PK(15)	CCL-33	Pig	Kidney	Epithelial	++	++	2.83	SP
MDCK	CCL-34	Dog	Kidney	Epithelial	++	+++	17	SP
RK13	CCL-37	Rabbit	Kidney	Epithelial	-/+	+++	0.032	NP
C6	CCL-107	Rat	Glial tumor	Fibroblast	++	+++	4.25	SP
Vero	CRL-81	African green monkey	Kidney	Epithelial	+++	+++	94	P
CEF	Primary	Chicken embryo	Assorted	Fibroblast	+++	+++	92.5	P
NIH/3T3	CRL-1658	Mouse	Embryo	Fibroblast	+++	+++	4.44	SP

^a Virus spread was visualized by immunostaining after 48 h. (–) No stained cells; (+) foci of 1–4 stained cells; (++) foci of 5–25 stained cells; (+++) foci of >25 stained cells (Carroll and Moss, 1997). Cells were infected with an MOI of 0.01.

^b Virus replication (fold increase in virus titer) determined by dividing the virus yield at 72 h by the input titer. Cell lines were therefore categorized into permissive (P, >25-fold increase), semi-permissive (SP, 1–25-fold increase) and non-permissive (NP, <1-fold increase) (Carroll and Moss, 1997). Cells were infected with an MOI of 0.05.

An immunostaining analysis on cells infected with MVTT₂-GFP was conducted to determine the degree of cell-to-cell spread in order to further understand the underlined mechanism of reduced viral growth capacity. All 13 mammalian cell lines and CEF became infected with MVTT₂-GFP. The infection was indicated by the expression of specific antigens stained

with a polyclonal anti-vaccinia serum in infected cells (Fig. 2). When compared with the parental VTT strain, the MVTT₂-GFP spread well in cell lines including COS-7, Vero, WISH, BHK-21, MRC-5, NIH/3T3 and CEF. MVTT₂-GFP, however, displayed restricted spread in several cell lines including MDCK, C6 and 293T (Fig. 2). The fewer number of positive cells found in

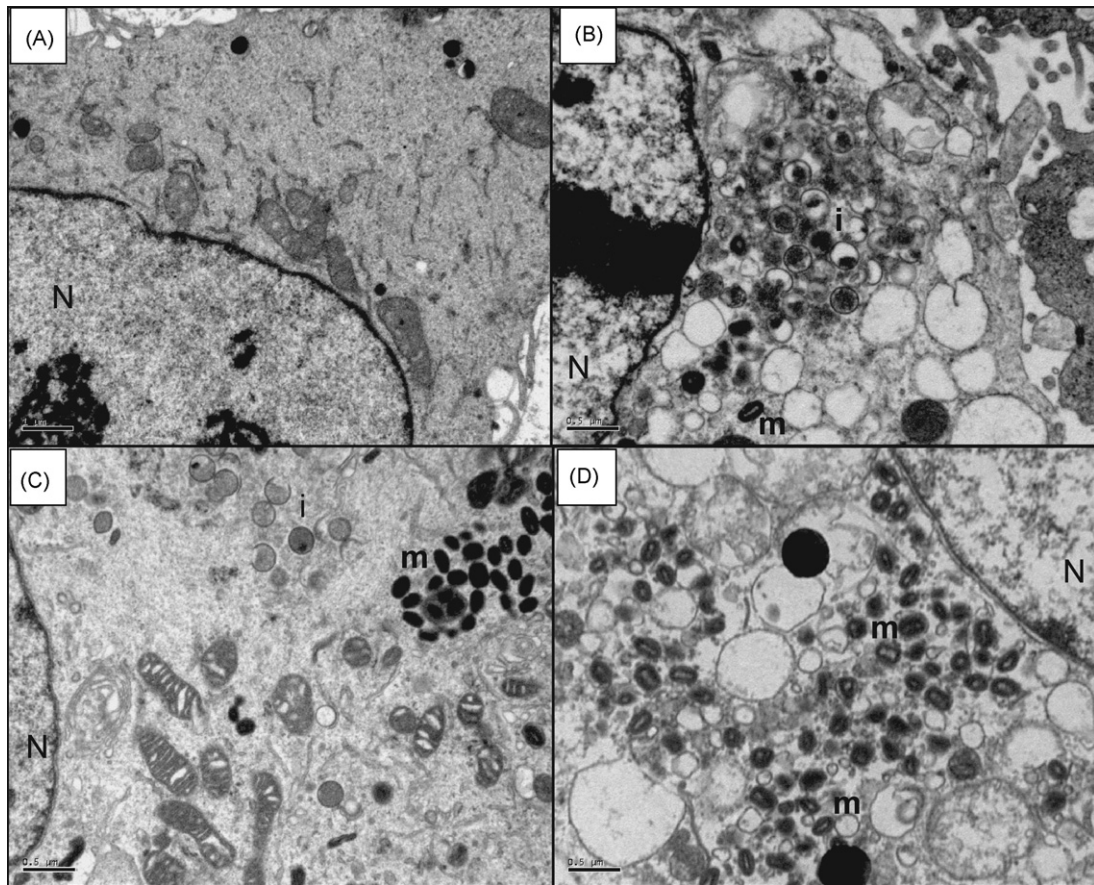


Fig. 3. Electron microscopy (EM) analysis of RK13 (A and C) and HeLa (B and D) cells infected with MVTT₂-GFP (A and B) or VTT (C and D). The cells were infected with an MOI of 5 and subjected to EM analysis at 16 h p.i. The individual letters indicate different observed structures including: i, typical immature virion; m, brick-shaped mature virion; N, nucleoid. The black bars shown at the bottom left corner of each photo represent the equivalent physical sizes: 1 μm in A and 0.5 μm in B, C and D.

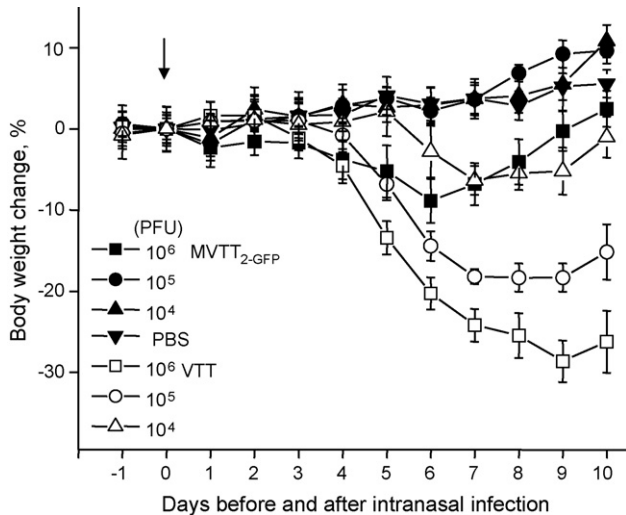


Fig. 4. Comparison of the virulence of MVTT_{2-GFP} or VTT in mice by the assessment of body weight loss of infected animals. Groups of five BALB/c mice were infected intranasally with different doses of MVTT_{2-GFP}, VTT or PBS on day 0 (arrow). The weight loss over time is represented by the mean values of each group of animals p.i. The error bar indicates the experimental variation. The difference between MVTT_{2-GFP} and VTT at each of the three dose groups is statistically significant ($p < 0.05$, ANOVA).

each foci at 48 h p.i. indicates that the viral spread via cell-to-cell contact was less efficient. The reduced spread capacity of MVTT_{2-GFP} via cell-to-cell contact was likely a reason for the decreased replication kinetics in these cell lines.

3.3. Virus morphogenesis in RK13 and HeLa cells

To understand further the viral life cycle in RK13 and HeLa cells, MVTT_{2-GFP} and the parental VTT were subjected to the EM examination. By examining the morphology of newly produced viral particles, the stage of viral replication blockade in these cell lines was expected to be resolved. RK13 and HeLa cells were analyzed around 16 h p.i. (Fig. 3). As shown in Fig. 3A, neither intracellular mature virions (IMVs) nor immature virions (IVs) were found in RK13 cells infected with MVTT_{2-GFP}. However, HeLa cells showed different results

(Fig. 3B). Immature virions were often found in the cytoplasm of HeLa cells whereas few IMVs could be identified. As controls, both cell lines produced the majority of the viral particles with characteristic brick-shape of IMV in their cytoplasm after they were infected with the parental VTT (Fig. 3C and D). Therefore, the data indicated that the aborted viral replication in these two cell lines was likely due to blockades at different stages of viral replication, before the assembly of immature virions in RK13 cells and around the maturation time of IMV morphogenesis in HeLa cells.

3.4. In vivo virulence of MVTT_{2-GFP} in mice

The *in vivo* virulence of MVTT_{2-GFP} was evaluated in inbred BALB/c mice using methods as described previously (Lee et al., 1992; Betakova et al., 2000; Zhang et al., 2000). The viral virulence was first determined by the daily measurement of body weight change for a period of 10 days after the animals were inoculated with the virus via the intranasal route. Five mice were tested for each of the three dose groups: 10⁴, 10⁵ and 10⁶ PFU per mouse. As shown in Fig. 4, none of the infected mice died during the experiment. Although mice infected with MVTT_{2-GFP} showed less than 10% of weight loss over 7 days in the high dose group (10⁶ PFU), the virus is significantly less virulent than the parental VTT, which resulted in about 30% weight loss during the same period of time (Fang et al., 2005). Interestingly, the body weight change in mice infected with 10⁶ PFU of MVTT_{2-GFP} was similar to that of mice that were given 10⁴ PFU of parental VTT (Fig. 4). These results suggest that MVTT_{2-GFP} was attenuated for about 100-fold in this setting.

Subsequently, the neurovirulence of MVTT_{2-GFP} was evaluated by infecting BALB/c mice via the intracranial route. The neurovirulence in infected mice was determined by measuring 50% of the intracranial lethal infectious dose (ICLD₅₀). Six 4-week-old mice per group were inoculated with a series of five-fold diluted viruses starting from 3×10^6 PFU. By counting the number of dead animals daily (Fig. 5), the ICLD₅₀ was calculated using the Reed–Muench method (Reed and Muench, 1938). ICLD₅₀ of MVTT_{2-GFP} was 1.05×10^6 PFU.

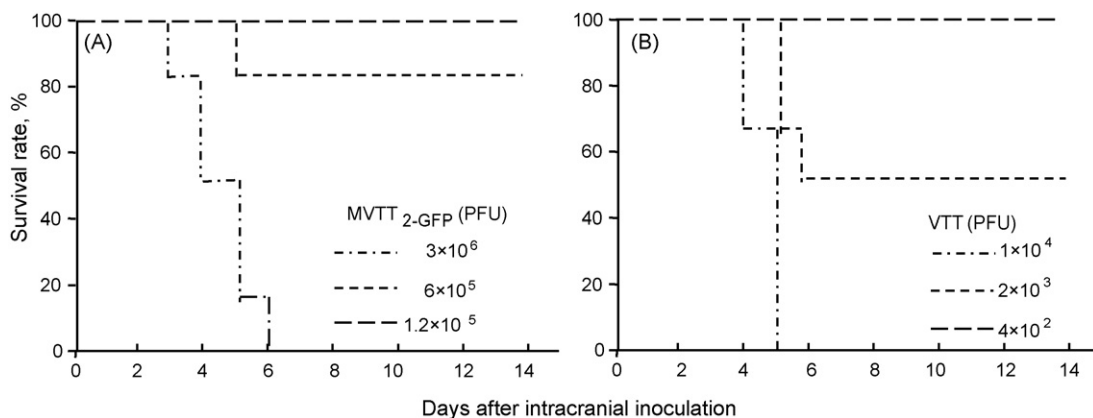


Fig. 5. Dose-dependent mortality after injection with vaccinia virus MVTT_{2-GFP} (A) or VTT (B). Groups of six BALB/c mice were infected intracranially with different doses of MVTT_{2-GFP} or VTT. The animals were observed for the percentage of survival up to 14 days p.i.

Since the ICLD₅₀ of the parent VTT is 3.1×10^3 PFU, these results demonstrated that MVTT_{2-GFP} has been attenuated for about 340-fold.

3.5. Stability of GFP gene in MVTT_{2-GFP} genome

A series of passages of the virus were conducted in Vero cells in order to determine whether MVTT_{2-GFP} could be developed as a novel vaccine vector. After 10 rounds of passage, anti-vaccinia antibodies were used to stain the infected foci. By evaluating 98 foci, all displayed GFP expression (data not shown). This finding indicates that the foreign GFP gene is stable in the genome of MVTT_{2-GFP}. This pure GFP-expressing virus may enable the generation of reporter gene-naïve MVTT_{2-GFP}-based vaccines through reverse GFP selection. This is crucial for eliminating the redundant reporter gene in vaccines for human use.

3.6. Immunogenicity of MVTT_{2-GFP}

Groups of BALB/c mice were inoculated with the virus to evaluate how well MVTT_{2-GFP} could be used as a vaccine vector. The immune responses against the foreign protein GFP were subsequently determined by an ELISA against purified recombinant EGFP. MVTT_{2-GFP} induced a GFP specific antibody response. The response was detected after the first immunization with an average serum reciprocal titer of 250 (Fig. 6A). After the second immunization, there was a significant boost effect. The average antibody response to GFP was doubled and the average serum reciprocal titer reached 700. The level of this antibody response was similar to that induced by a control recombinant VTT_{GFP} (Fig. 6A). Moreover, the cell-mediated immune response to GFP was also detected by an ELISpot assay. Approximately 350 spot-forming cells (SFC) per million splenocytes were detected against a specific CTL epitope HYLSTQSAL (Fig. 6B). Immunization with VTT_{GFP} also induced a

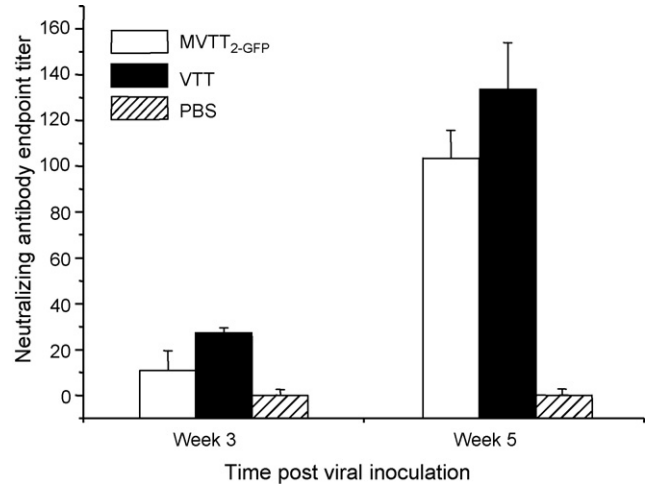


Fig. 7. Neutralizing antibody responses elicited against VTT in mice immunized with MVTT_{2-GFP} or VTT. Serum samples collected at weeks 3 and 5 p.i. were tested. Antibody titers were defined as the reciprocal of serum dilution that reduced viral plaques by 50%. Bars represent the average values of three samples (\pm standard deviations). Serum samples from pre-immunized or mock-immunized animals were included as background controls. This experiment was repeated twice with similar results obtained.

cell-mediated immune response to GFP but at a slightly lower level. In contrast, immunization with wild type VTT or placebo did not induce any immune responses to GFP. These data indicate that MVTT_{2-GFP} is capable of eliciting specific immune responses against foreign antigens *in vivo* likely with a comparable potency of VTT_{GFP}.

In addition to the immune responses against GFP, the neutralizing antibody titer against the parental vector VTT was also evaluated. Consistent with the antibody response against GFP, the level of neutralizing antibody response against VTT was boosted significantly after the second immunization (Fig. 7). A slightly higher level of neutralizing antibodies was induced

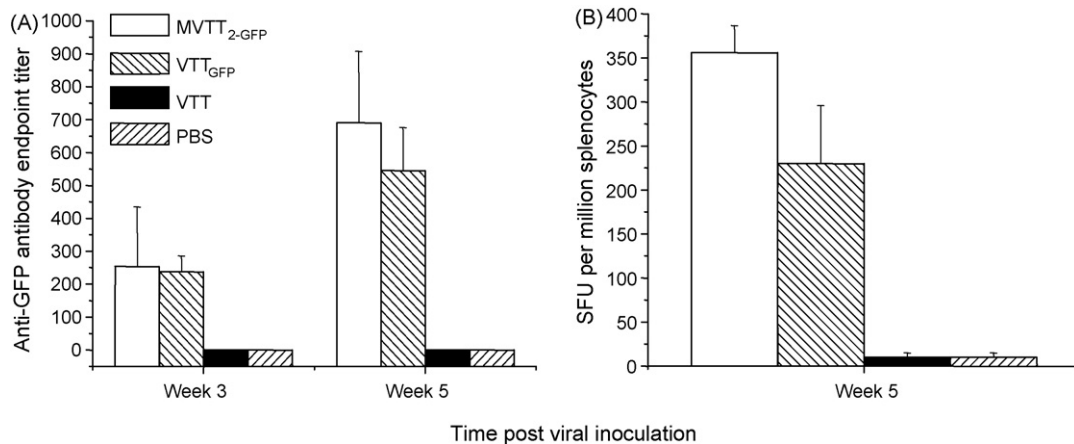


Fig. 6. Humoral and cell-mediated immune responses elicited against GFP in mice immunized with MVTT_{2-GFP}. Mice were intramuscularly inoculated with 10^5 PFU of MVTT_{2-GFP} or VTT and boosted at a 3-week interval. Mice that received PBS served as controls. (A) Sera were tested for specific IgG antibodies against GFP by ELISA at day 0, weeks 3 and 5 p.i. Responses in naïve (week 0) animals are not shown as the absorbance readouts of those samples fell below the cutoff values that were used for the determination of antibody titer. The differences between MVTT_{2-GFP} and VTT at test time points are statistically significant ($p < 0.01$). (B) The number of IFN- γ -secreting CD8⁺ T cells specific for a GFP epitope HYLSTQSAL was determined by an ELISpot assay. Bars represent the average values of three samples (\pm standard deviations). The difference between MVTT_{2-GFP} and VTT at week 5 p.i. is statistically significant ($p < 0.01$). These experiments were repeated twice with similar results obtained.

by VTT than by MVTT_{2-GFP}. This difference, however, did not reach statistical significance. Therefore, the removal of the M1L-K2L genes did not seem to affect the major neutralizing determinants of vaccinia virus.

4. Discussion

MVTT_{2-GFP} displayed distinct biological properties when compared with the parental VTT. First, MVTT_{2-GFP} displayed a reduced replication capacity in two human cell lines, HeLa and 293T, as well as in animal cell lines including PK(15), RK13, C6 and MDCK. These phenotypic changes were found to be likely related to the reduced replication and cell-to-cell spread capacity of MVTT_{2-GFP}. The RK13 and HeLa cell lines became non-permissive to MVTT_{2-GFP} infection. The EM examination indicated that the life cycle of MVTT_{2-GFP} was significantly blocked at a relatively early stage before the assembly of immature virions in RK13 cells but at a stage of virion maturation in HeLa cells. Second, MVTT_{2-GFP} is much less virulent as determined by two separated experiments. About 10% body weight loss was found at day 7 p.i. in mice that received the high dose (10⁶ PFU) of MVTT_{2-GFP} via the intranasal route of inoculation. Since 10⁴ PFU of the parental VTT caused a similar level of body weight loss, MVTT_{2-GFP} was likely attenuated by about 100-fold. Moreover, MVTT_{2-GFP} displayed an increase of intracranial LD₅₀ in mice via the intracranial inoculation that was 340 times greater than the parental VTT. These findings indicate that MVTT_{2-GFP} is an attenuated vaccinia strain. This attenuation is essential for its future development as a safe vaccine vector (Lee et al., 1992; Moss, 1996; Tartaglia et al., 1992). This attenuation, however, does not seem to affect the expression of the foreign antigens GFP in various cell types infected individually. This property is a prerequisite for a vaccinia-based vaccine vector. The GFP gene in MVTT_{2-GFP} may serve either as a reverse selection marker for vaccine construction or as a reporter in a neutralization assay.

The phenotypic changes observed in MVTT_{2-GFP} are related to the loss of the M1L-K2L genes in the VTT genome. Previous studies revealed that VTT displays genetic features distinct from other vaccinia viruses (Hou et al., 1985; Tsao et al., 1986). These genetic features led to distinct biological characteristics of VTT when compared to vaccinia WR strain (Fang et al., 2005). In this study, four vaccinia genes including three early genes, M1L, M2L and K1L, as well as one intermediate gene, K2L were removed from the VTT genome to generate MVTT_{2-GFP}. These four genes have not been found associated with the virulence of a vaccinia virus *in vivo* before. Of them, K1L is a previously defined host range gene that is essential for vaccinia viral replication in RK13 cells (Chung et al., 1997; Perkus et al., 1990; Ramsey-Ewing and Moss, 1996). Consistent with previous findings for VVdelK1L and MVA, MVTT_{2-GFP} replicated poorly also in RK13 cells. Since the K1L gene inhibits the apoptosis of RK13 cells and host NF-kappaB activation (Shisler and Jin, 2004), a separate study is necessary to address whether or not the K1L gene of VTT alone would have similar biological functions. The K2L gene encodes a serine protease inhibitor which inhibits cell–cell fusion (Law and Smith, 1992; Turner and Moyer, 1995;

Zhou et al., 1992). The deletion of K2L alone did not change virus replication *in vitro* as well as the virulence and immunogenicity *in vivo* (Law and Smith, 1992; Turner and Moyer, 1995; Zhou et al., 1992). The loss of K2L gene in MVTT_{2-GFP} does not induce cell–cell fusion in every cell type tested (Fig. 3). Consistent with previous findings, the property of enhanced cell fusion does not increase the virulence of MVTT_{2-GFP} *in vivo* because the virus was attenuated instead. To date, the biological functions of vaccinia M1L and M2L genes remain not clear. M1L was suggested previously to function as an ankyrin-like protein (Shchelkunov et al., 1993). At this stage, although the loss of M1L-K2L genes has resulted in the formation of an attenuated MVTT_{2-GFP}, whether or not each of the four genes has contributed to the phenotypic changes remains exclusive. Further analysis of each individual gene is required to obtain a definitive answer. Nevertheless, the loss of the M1L-K2L genes does not affect the replication of MVTT_{2-GFP} in Vero and CEF cells. Since other attenuated viruses (e.g. MVA) rely solely on CEF cells for production, the growth property of MVTT_{2-GFP} in Vero cells may offer an advantage for future manufacture because Vero is one of the WHO and FDA approved cell lines for vaccine production. As for the development of a human vaccine vector, it is anticipated that the further inactivation of additional VTT genes involved in host cell range (e.g. C7L) and in viral virulence (e.g. hemagglutinin, TK, etc.) would be necessary (Antoine et al., 1996; Shida et al., 1988).

MVTT_{2-GFP} elicits specific immune response against the foreign antigen GFP. To use an attenuated vaccinia virus as a live vaccine vector, one critical question is whether or not this virus is still immunogenic for inducing specific immune response against foreign antigens. To address this question, the immune response against the vector and foreign antigen was determined. With a suboptimal dose of 10⁵ PFU, MVTT_{2-GFP} and VTT induced similar levels of neutralizing antibody responses to VTT (Fig. 7). GFP-specific humoral and cell-mediated immune responses were detected. The levels of these responses are comparable to those induced by a VTT_{GFP} virus. Since GFP is apparently stable in the genome of MVTT_{2-GFP}, this virus can be developed further to become a live vaccine vector for infectious pathogens or tumors. In this case, the genes encoding the pathogen or tumor specific antigens can be placed in the genomic location of the GFP gene. In future studies, a comparative study using MVTT_{2-GFP} and other vaccinia strains (e.g. MVA) is essential to reveal the fundamental differences or advantages of these systems to serve as vaccine vectors. Considering that MVTT_{2-GFP} remains replication-competent in several mammalian cells tested, a correlation between dosing and vaccine efficacy needs to be evaluated, as would the non-invasive mucosal routes of vaccination. The latter is critical because the inoculation of vaccinia-based vaccine via mucosal route can induce immune response against foreign genes even in the presence of preexisting anti-vector immunity (Belyakov et al., 1999). It was demonstrated that mucosal inoculation of vaccinia-immune BALB/c mice with recombinant vaccinia expressing HIV-1 gp160 induced specific serum antibody and strong HIV-1-specific cytotoxic T lymphocyte responses. For live viral vaccine vectors, it is essential to evade the preexisting anti-vector immu-

nity and to stimulate robust immune responses at the site of viral transmission (e.g. influenza and HIV-1). For individuals who did not receive smallpox vaccination (born after 1980), the preexisting anti-vector immunity is not an issue.

Acknowledgements

This study was supported by the National Basic Research Program of China (973 project 2006CB504208 and sub-project 2005CB522903) (to ZC) and the Special Fund of Wuhan University to the Modern Virology Research Center. We also acknowledge partial financial supports by the NIH R01 grant HL080211-01 (to ZC).

References

- Antoine, G., Scheifflinger, F., Dorner, F., Falkner, F.G., 1998. The complete genomic sequence of the modified vaccinia Ankara strain: comparison with other orthopoxviruses. *Virology* 244, 365–396.
- Antoine, G., Scheifflinger, F., Holzer, G., Langmann, T., Falkner, F.G., Dorner, F., 1996. Characterization of the vaccinia MVA hemagglutinin gene locus and its evaluation as an insertion site for foreign genes. *Gene* 177, 43–46.
- Barouch, D.H., Santra, S., Kuroda, M.J., Schmitz, J.E., Plishka, R., Buckler-White, A., Gaitan, A.E., Zin, R., Nam, J.H., Wyatt, L.S., Lifton, M.A., Nickerson, C.E., Moss, B., Montefiori, D.C., Hirsch, V.M., Letvin, N.L., 2001. Reduction of simian-human immunodeficiency virus 89.6P viremia in rhesus monkeys by recombinant modified vaccinia virus Ankara vaccination. *J. Virol.* 75, 5151–5158.
- Belyakov, I.M., Moss, B., Strober, W., Berzofsky, J.A., 1999. Mucosal vaccination overcomes the barrier to recombinant vaccinia immunization caused by preexisting poxvirus immunity. *Proc. Natl. Acad. Sci. U.S.A.* 96, 4512–4517.
- Betakova, T., Wolffe, E.J., Moss, B., 2000. The vaccinia virus A14.5L gene encodes a hydrophobic 53-amino-acid virion membrane protein that enhances virulence in mice and is conserved among vertebrate poxviruses. *J. Virol.* 74, 4085–4092.
- Bisht, H., Roberts, A., Vogel, L., Bukreyev, A., Collins, P.L., Murphy, B.R., Subbarao, K., Moss, B., 2004. Severe acute respiratory syndrome coronavirus spike protein expressed by attenuated vaccinia virus protectively immunizes mice. *Proc. Natl. Acad. Sci. U.S.A.* 101, 6641–6646.
- Carroll, M.W., Moss, B., 1997. 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* 238, 198–211.
- Chakrabarti, S., Sisler, J.R., Moss, B., 1997. Compact, synthetic, vaccinia virus early/late promoter for protein expression. *Biotechniques* 23, 1094–1097.
- Chen, Z., Zhang, L., Qin, C., Ba, L., Yi, C.E., Zhang, F., Wei, Q., He, T., Yu, W., Yu, J., Gao, H., Tu, X., Gettie, A., Farzan, M., Yuen, K.Y., Ho, D.D., 2005. Recombinant modified vaccinia virus Ankara expressing the spike glycoprotein of severe acute respiratory syndrome coronavirus induces protective neutralizing antibodies primarily targeting the receptor binding region. *J. Virol.* 79, 2678–2688.
- Chung, C.S., Vasilevskaya, I.A., Wang, S.C., Bair, C.H., Chang, W., 1997. Apoptosis and host restriction of vaccinia virus in RK13 cells. *Virus Res.* 52, 121–132.
- Cosma, A., Nagaraj, R., Buhler, S., Hinkula, J., Busch, D.H., Sutter, G., Goebel, F.D., Erfle, V., 2003. Therapeutic vaccination with MVA-HIV-1 nef elicits Nef-specific T-helper cell responses in chronically HIV-1 infected individuals. *Vaccine* 22, 21–29.
- De Waal, L., Wyatt, L.S., Yuksel, S., van Amerongen, G., Moss, B., Niesters, H.G., Osterhaus, A.D., de Swart, R.L., 2004. Vaccination of infant macaques with a recombinant modified vaccinia virus Ankara expressing the respiratory syncytial virus F and G genes does not predispose for immunopathology. *Vaccine* 22, 923–926.
- Degano, P., Schneider, J., Hannan, C.M., Gilbert, S.C., Hill, A.V., 1999. Gene gun intradermal DNA immunization followed by boosting with modified vaccinia virus Ankara: enhanced CD8+ T cell immunogenicity and protective efficacy in the influenza and malaria models. *Vaccine* 18, 623–632.
- Drillien, R., Koehren, F., Kirn, A., 1981. Host range deletion mutant of vaccinia virus defective in human cells. *Virology* 111, 488–499.
- Fang, Q., Yang, L., Zhu, W., Liu, L., Wang, H., Yu, W., Xiao, G., Tien, P., Zhang, L., Chen, Z., 2005. Host range, growth property, and virulence of the smallpox vaccine: vaccinia virus Tian Tan strain. *Virology* 335, 242–251.
- Gambotto, A., Dworacki, G., Cicinnati, V., Kenniston, T., Steitz, J., Tuting, T., Robbins, P.D., DeLeo, A.B., 2000. Immunogenicity of enhanced green fluorescent protein (EGFP) in BALB/c mice: identification of an H2-Kd-restricted CTL epitope. *Gene Ther.* 7, 2036–2040.
- Gillard, S., Spehner, D., Drillien, R., Kirn, A., 1986. Localization and sequence of a vaccinia virus gene required for multiplication in human cells. *Proc. Natl. Acad. Sci. U.S.A.* 83, 5573–5577.
- Goonetilleke, N., Moore, S., Dally, L., Winstone, N., Ceber, I., Mahmoud, A., Pinheiro, S., Gillespie, G., Brown, D., Loach, V., Roberts, J., Guimaraes-Walker, A., Hayes, P., Loughran, K., Smith, C., De Bont, J., Verlinde, C., Vooijs, D., Schmidt, C., Boaz, M., Gilmour, J., Fast, P., Dorrell, L., Hanke, T., McMichael, A.J., 2006. Induction of multifunctional human immunodeficiency virus type 1 (HIV-1)-specific T cells capable of proliferation in healthy subjects by using a prime-boost regimen of DNA- and modified vaccinia virus Ankara-vectored vaccines expressing HIV-1 Gag coupled to CD8+ T-cell epitopes. *J. Virol.* 80, 4717–4728.
- Hou, Y.T., Yang, X.K., Hu, Y.W., 1985. Variation in the HindIII restriction fragments of DNA from the Chinese Tian Tan strain of vaccinia virus. *J. Gen. Virol.* 66 (Pt 8), 1819–1823.
- Hsu, C., Boysen, M., Gritton, L.D., Frosst, P.D., Nemerow, G.R., Von Seggern, D.J., 2005. In vitro dendritic cell infection by pseudotyped adenoviral vectors does not correlate with their in vivo immunogenicity. *Virology* 332, 1–7.
- Jin, Q., Chen, L.H., Chen, S.X., Huang, J., Feng, Z.H., Yuan, J.S., Jin, D.Y., Bai, H.D., Hou, Y.D., 1997. Characterization of the complete genomic sequence of the vaccinia virus Tian Tan strain. *Sci. China (Series)* 27 (6), 562–567.
- Jing, L., Chong, T.M., McClurkan, C.L., Huang, J., Story, B.T., Koelle, D.M., 2005. Diversity in the acute CD8 T cell response to vaccinia virus in humans. *J. Immunol.* 175, 7550–7559.
- Law, K.M., Smith, G.L., 1992. A vaccinia serine protease inhibitor which prevents virus-induced cell fusion. *J. Gen. Virol.* 73 (Pt 3), 549–557.
- Lee, M.S., Roos, J.M., McGuigan, L.C., Smith, K.A., Cormier, N., Cohen, L.K., Roberts, B.E., Payne, L.G., 1992. Molecular attenuation of vaccinia virus: mutant generation and animal characterization. *J. Virol.* 66, 2617–2630.
- Meyer, H., Sutter, G., Mayr, A., 1991. Mapping of deletions in the genome of the highly attenuated vaccinia virus MVA and their influence on virulence. *J. Gen. Virol.* 72 (Pt 5), 1031–1038.
- Moss, B., 1996. Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety. *Proc. Natl. Acad. Sci. U.S.A.* 93, 11341–11348.
- Moss, B., Carroll, M.W., Wyatt, L.S., Bennink, J.R., Hirsch, V.M., Goldstein, S., Elkins, W.R., Fuerst, T.R., Lifson, J.D., Piatak, M., Restifo, N.P., Overwijk, W., Chamberlain, R., Rosenberg, S.A., Sutter, G., 1996. Host range restricted, non-replicating vaccinia virus vectors as vaccine candidates. *Adv. Exp. Med. Biol.* 397, 7–13.
- Mwau, M., Ceber, I., Sutton, J., Chikoti, P., Winstone, N., Wee, E.G., Beattie, T., Chen, Y.H., Dorrell, L., McShane, H., Schmidt, C., Brooks, M., Patel, S., Roberts, J., Conlon, C., Rowland-Jones, S.L., Bwayo, J.J., McMichael, A.J., Hanke, T., 2004. A human immunodeficiency virus 1 (HIV-1) clade A vaccine in clinical trials: stimulation of HIV-specific T-cell responses by DNA and recombinant modified vaccinia virus Ankara (MVA) vaccines in humans. *J. Gen. Virol.* 85, 911–919.
- Olszewska, W., Suezter, Y., Sutter, G., Openshaw, P.J., 2004. Protective and disease-enhancing immune responses induced by recombinant modified vaccinia Ankara (MVA) expressing respiratory syncytial virus proteins. *Vaccine* 23, 215–221.
- Paoletti, E., 1996. Applications of pox virus vectors to vaccination: an update. *Proc. Natl. Acad. Sci. U.S.A.* 93, 11349–11353.
- Perkus, M.E., Goebel, S.J., Davis, S.W., Johnson, G.P., Limbach, K., Norton, E.K., Paoletti, E., 1990. Vaccinia virus host range genes. *Virology* 179, 276–286.

- Ramsey-Ewing, A.L., Moss, B., 1996. Complementation of a vaccinia virus host-range K1L gene deletion by the nonhomologous CP77 gene. *Virology* 222, 75–86.
- Reed, L.J., Muench, H., 1938. A simple method of estimating fifty percent endpoints. *Am. J. Hyg.* 27, 493–497.
- Santra, S., Seaman, M.S., Xu, L., Barouch, D.H., Lord, C.I., Lifton, M.A., Gorgone, D.A., Beaudry, K.R., Svehla, K., Welcher, B., Chakrabarti, B.K., Huang, Y., Yang, Z.Y., Mascola, J.R., Nabel, G.J., Letvin, N.L., 2005. Replication-defective adenovirus serotype 5 vectors elicit durable cellular and humoral immune responses in nonhuman primates. *J. Virol.* 79, 6516–6522.
- Shchelkunov, S.N., Blinov, V.M., Sandakhchiev, L.S., 1993. Ankyrin-like proteins of variola and vaccinia viruses. *FEBS Lett.* 319 (1/2), 163–165.
- Shida, H., Hinuma, Y., Hatanaka, M., Morita, M., Kidokoro, M., Suzuki, K., Maruyama, T., Takahashi-Nishimaki, F., Sugimoto, M., Kitamura, R., et al., 1988. Effects and virulences of recombinant vaccinia viruses derived from attenuated strains that express the human T-cell leukemia virus type I envelope gene. *J. Virol.* 62, 4474–4480.
- Shisler, J.L., Jin, X.L., 2004. The vaccinia virus K1L gene product inhibits host NF-kappaB activation by preventing IkappaBalpha degradation. *J. Virol.* 78, 3553–3560.
- Sutter, G., Moss, B., 1992. Nonreplicating vaccinia vector efficiently expresses recombinant genes. *Proc. Natl. Acad. Sci. U.S.A.* 89, 10847–10851.
- Sutter, G., Wyatt, L.S., Foley, P.L., Bennink, J.R., Moss, B., 1994. A recombinant vector derived from the host range-restricted and highly attenuated MVA strain of vaccinia virus stimulates protective immunity in mice to influenza virus. *Vaccine* 12, 1032–1040.
- Tartaglia, J., Perkus, M.E., Taylor, J., Norton, E.K., Audonnet, J.C., Cox, W.I., Davis, S.W., van der Hoeven, J., Meignier, B., Riviere, M., et al., 1992. NYVAC: a highly attenuated strain of vaccinia virus. *Virology* 188, 217–232.
- Tsao, H., Ren, G.F., Chu, C.M., 1986. Gene coding for the late 11,000-dalton polypeptide of the Tian Tan strain of vaccinia virus and its 5'-flanking region: nucleotide sequence. *J. Virol.* 57, 693–696.
- Turner, P.C., Moyer, R.W., 1995. Orthopoxvirus fusion inhibitor glycoprotein SPI-3 (open reading frame K2L) contains motifs characteristic of serine proteinase inhibitors that are not required for control of cell fusion. *J. Virol.* 69, 5978–5987.
- Wolffe, E.J., Moore, D.M., Peters, P.J., Moss, B., 1996. Vaccinia virus A17L open reading frame encodes an essential component of nascent viral membranes that is required to initiate morphogenesis. *J. Virol.* 70, 2797–2808.
- Wyatt, L.S., Shors, S.T., Murphy, B.R., Moss, B., 1996. Development of a replication-deficient recombinant vaccinia virus vaccine effective against parainfluenza virus 3 infection in an animal model. *Vaccine* 14, 1451–1458.
- Wyatt, L.S., Whitehead, S.S., Venanzi, K.A., Murphy, B.R., Moss, B., 1999. Priming and boosting immunity to respiratory syncytial virus by recombinant replication-defective vaccinia virus MVA. *Vaccine* 18, 392–397.
- Zhang, W.H., Wilcock, D., Smith, G.L., 2000. Vaccinia virus F12L protein is required for actin tail formation, normal plaque size, and virulence. *J. Virol.* 74, 11654–11662.
- Zhou, J., Sun, X.Y., Fernando, G.J., Frazer, I.H., 1992. The vaccinia virus K2L gene encodes a serine protease inhibitor which inhibits cell–cell fusion. *Virology* 189, 678–686.