Antiviral Research 96 (2012) 324–332

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Characterization of an attenuated TE3L-deficient vaccinia virus Tian Tan strain

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ARTICLE INFO

Article history:
Received 5 April 2012
Revised 7 October 2012
Accepted 8 October 2012
Available online 17 October 2012

Keywords:
Attenuated vaccinia virus vector
Tian Tan strain
TE3L
Virulence
Immunogenicity

ABSTRACT

An attenuated vaccinia virus (VACV), TE3L⁻VTT, was evaluated for virulence and safety to determine its potential use as a vaccine or as a recombinant virus vector to express foreign genes. The virulence of TE3L⁻VTT was compared with that of the wild-type VTT both *in vivo* and *in vitro*. The humoral and cellular immune responses were detected in a mouse model to test the vaccine efficacy of the TE3L mutant. The results suggested that deletion of the TE3L gene decreased the virulence and neurovirulence significantly in mice and rabbit models, yet retained the immunogenicity. Thus, the deletion of TE3L improved the safety of the VTT vector; this approach may yield a valuable resource for studies of recombinant VACV-vectored vaccines.

1. Introduction

Vaccinia virus is a double-stranded DNA virus that replicates and multiplies in the cytoplasm of infected cells using both virally encoded proteins and resources of the host cells. The attenuated form of vaccinia virus (VACV) has been used as a vaccine against smallpox to successfully eradicate this human disease. However, it has been reported that the use of VACV has resulted in several adverse events, which include encephalitis, eczema after vaccination, generalized and progressive poxvirus infections, as well as some less severe reactions (Wollenberg and Engler, 2004). Furthermore, cardiac complications have been reported to occur after vaccination (Cassimatis et al., 2004). Increased numbers of individuals have impaired immune functions due to organ transplantation, human immunodeficiency virus (HIV) infection and cancer treatments, which may affect the development and clinical application of VACV or VACV-based vaccines in the future. All these consequences mean that there is a need to develop a safer VACV vector.

VACV has widespread application in biological and medical investigations, therefore scientific researchers have committed considerable efforts to study attenuated VACV vectors in order to overcome potential adverse reactions following vaccination. Some attenuated strains have been obtained so far, such as the modified Ankara strain (MVA) (Coulibaly et al., 2005; Meyer et al., 1991; Nigam et al., 2007), NYVAC (Tartaglia et al., 1992), the Lister mutant strain of VACV (Hiley et al., 2010; Tysome et al., 2009), Lister clone 16m8 (LC16m8) (Kenner et al., 2006) and Dairen I strain (Someya et al., 2004). The MVA, LC16m8 and Dairen I strain were obtained by passage in alternative hosts, while the third-generation attenuated strains, NYVAC and the Lister mutant, were generated by deletion of certain genes (Jacobs et al., 2009; Rosenwirth et al., 2001). Most of these vectors are replication-defective viruses in most mammalian cells, except for LC16m8 which has a limited capacity to replicate in human cells. Although non-replicating virus vectors have the advantage of increased safety, the use of high doses and multiple immunizations is generally required to induce a strong immune response (Meseda et al., 2005).

The *VACV E3L* gene, a host range and virulence gene, encodes a protein that inhibits the innate immune response (Beattie et al., 1996; Langland and Jacobs, 2002; Vijaysri et al., 2008). Recently, a study also shows that the amino terminus of the vaccinia virus E3L protein is necessary to inhibit the interferon response (White and Jacobs, 2012). We have noted previously that the full E3L

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http://dx.doi.org/10.1016/j.antiviral.2012.10.002

deletion mutation in the Copenhagen, Western Reserve (WR) and NYCBH strains of VACV are highly attenuated and more appropriate for use in humans (Fenner et al., 1988; Jentarra et al., 2008; Vijaysri et al., 2008). However, currently there are no reports of vaccinia virus Tian Tan strain deleted TE3L gene, and the pathogenesis and immunogenicity of TianTan strain vaccinia virus with deletion of TE3L (TE3L⁻VTT) are not known. To test the efficacy of the TE3L mutation in the TianTan strain, we constructed and recovered the mutant strain of VTT by deletion of the TE3L region, which has been found to contribute to pathogenesis and is necessary for full virulence in mouse models. The genetic stability of the mutant strain was analyzed by passage in baby hamster kidney (BHK)-21 cells. Importantly, we evaluated its safety and immunogenicity in both mouse and rabbit models. These systematic studies provided scientific support for the development of the mutant strain as a live virus vaccine vector with increased safety.

2. Materials and methods

2.1. Viruses, cells and animals

The vaccinia virus Tian Tan strain, kindly provided by Dr. Kuoshi Jin (Academy of Military Medical Sciences, Changchun, China), was propagated and titered in BHK-21 (baby hamster kidney) cells. BHK-21 and PK15 (porcine kidney) cells were maintained in minimal essential medium (MEM) with 5% fetal bovine serum (FBS) and 1% penicillin (10,000 U/mL)/streptomycin (10 mg/mL) solution (P/S). HeLa (human cervical carcinoma) and Vero (African green monkey kidney) cells were cultured in improved RPMI-1640 medium with 10% FBS and 1% P/S. The 293T (human embryo kidney) and MDCK (Madin–Darby canine kidney) cells were cultured with Dulbecco's modified Eagle's medium (DMEM) with 10% FBS and 1% P/S. All cells were incubated at 37 °C in 5% CO_2 in air. All media and supplements described above were obtained from HyClone (Beijing, China).

Female BALB/c mice were obtained from the Laboratory Animal Center of the Academy of Military Medical Sciences Beijing. Mice were 3 or 5 weeks of age when used for experiments. Female albino rabbits (1.7–3.0 kg) were provided by the Institute of Biological Products, Vaccine and Serum Institute of Changchun, China.

2.2. Construction of the recombinant TE3L⁻VTT virus

Homologous recombination was performed as described previously (Kan et al., 2012). Viruses were screened for fluorescent plaques under a fluorescence microscope and were obtained after 10 rounds of selection of GFP-positive plaques. Deletion of the *EGFP* gene from the recombinant virus was performed through the Cre/*loxP* recombination system. Mutant virus with the deleted *TE3L* gene (TE3L⁻VTT) was confirmed by PCR. The following primers were applied: *TE3L*-gene-specific primers (sense: 5′-GCAGAATCTAATGATGACGTAACCA-3′, antisense: 5′-GCGCCATGTCTAAAATCTATATCG-3′); *EGFP*-gene-specific primers (sense: 5′-ATCGATCGATGGTGAGCAAGGGCGAGGAG-3′, antisense: 5′-GCGTCGACTTACTTGTACAGCTCGTCCATG-3′).

2.3. Preparation of virus stocks

TE3L⁻VTT and VTT were prepared in BHK-21 cells and purified using the discontinuous sucrose gradient separation method (Chung et al., 2006). Briefly, 60%, 50%, 40% and 20% sucrose solutions were layered from the bottom of a centrifuge tube, the virus suspension was added to approximately 0.5–1 cm above the 20% sucrose layer. The final purified virus was obtained after centrifugation through the gradient at 26,000*g* for 50 min at 4 °C.

2.4. Virus morphological analysis

The morphology of purified TE3L⁻VTT and VTT viruses was observed under a TEM by two methods. The purified TE3L⁻VTT and VTT were observed directly after staining with 2% phosphotungstic acid at pH 1.0 (negative staining assay). To further assess the development of the viruses, their morphogenesis was observed in ultrathin sections. Briefly, monolayers of BHK-21 cells were infected with TE3L⁻VTT and VTT at a multiplicity of infection (MOI) of 10 pfu/cell. After 24 h of infection, the cells were scraped from the plate, centrifuged and the pellet was recovered and washed with PBS. The infected cells were fixed with pre-cooled 2.5% glutaraldehyde and then observed after routine processes of embedding, ultrathin sectioning and staining.

2.5. One-step growth curve

A one-step growth curve was performed to survey virus replication in BHK-21 and Vero cells as described previously (Embry et al., 2011). BHK-21 cells or Vero cells were infected (MOI = 5) with TE3L⁻VTT or VTT. The samples were harvested at 0, 16, 24, and 48 h post infection. Virus titers were enumerated on BHK-21 cells using crystal violet staining.

2.6. MTT assay

MTT assay was used to detect cell viability after infection through a succinic dehydrogenase activity assay in accordance with the method described by Li et al. (2006b). Vero, HeLa, MDCK, PK15 and BHK-21 cells were seeded in 96-well plates (1×10^4 cells/well) and cultured for 24 h before infection with 0.05 MOI/well of TE3L⁻VTT or VTT. Cell viability was measured every 12 h over a 72 h period by treating cells with MTT (5 mg/mL, 20 μL/well) and incubation for 4 h at 37 °C to allow MTT metabolism. Subsequently, the culture medium was removed and replaced with dimethylsulfoxide (100 μL/well) to dissolve the crystals. Finally, the absorbance was measured at 490 nm to indicate cell viability of different cell lines during different culture periods. Cell viability was calculated using the following formula: $OD_{experiment}/OD_{control} \times 100\%$, where non-infected cells were the control.

2.7. Weight changes in mice after infection

BALB/c mice at 5 weeks of age were challenged by intra-nasal infection (Vijaysri et al., 2008) with 20 μL of virus suspension in sterile PBS. The mice were anesthetized by intraperitoneal injection with 1% pentobarbital solution at approximately 50 μg/g body weight before intra-nasal infection. The groups of 10 mice each were set up as follows: 10^5 plaque forming units (pfu)/mouse of TE3L⁻VTT or VTT, 10^6 pfu/mouse of TE3L⁻VTT or VTT or 10^7 pfu/mouse of TE3L⁻VTT or VTT. Mock-infected mice received the same volume of sterile PBS without virus. All mice were monitored for clinical symptoms and weight changes for 25 consecutive days after challenge.

2.8. Intracranial 50% lethal infectious dose ($ICLD_{50}$) analysis

BALB/c mice at 3 weeks of age were infected by intracranial inoculation with 100 μL virus suspension (TE3L⁻VTT or VTT) in sterile PBS following anesthesia as described above. The groups were set up as described above. Mice inoculated with sterile PBS served as controls. Infected mice were monitored for mortality and general signs of disease over 14 consecutive days to construct a survival curve. The mice were also anesthetized before intracranial inoculation according to the method described above.

2.9. Virus induced skin lesions

The hair on the rabbit dorsal skin was removed with a depilatory. In briefly, the hair was firstly cut with curved scissor, and then the depilatory was coated on the skin for five minutes, then the hair gently scraped and washed thoroughly with sterile water. The virus suspension (100 μL in sterile PBS) was then inoculated intradermally at the prepared site. A rabbit was challenged with TE3L⁻VTT, VTT or PBS, respectively. Three injections of 10^5, 10^6 and 10^7 pfu of TE3L⁻VTT or VTT were given at multiple sites on each rabbit. Each dose of virus was repeated three times. The infected rabbits were observed every day until lesion resolution, and ulcerations and indurations were measured and recorded for analysis.

2.10. Vaccination of mice

Groups of three 6- to 8-week-old BALB/c mice were immunized intramuscularly twice with 1×10^6 pfu of TE3L⁻VTT or the wild-type strain VTT in 100 μL of PBS at weeks 0 and 3. Mock-infected mice received 100 μL PBS that contained no virus. Blood samples were collected 0, 7, 14, 21 and 30 days after the initial vaccination, followed by centrifugation at 3500 rpm for 10 min to separate out the sera from the red blood cells. Sera were stored at −70 °C for further testing. Splenocytes from mice 10 days post booster immunization were collected using methods described previously (Clark et al., 2006; Rehm et al., 2010).

2.11. Flow cytometry

Flow cytometry was performed as described previously (Li et al., 2006a; Rehm and Roper, 2011). In total, 1×10^6 splenocytes were washed with fluorescence-activated cell sorter (FACS) buffer, and stained with PerCP–Cy5.5-labeled anti-mouse CD3, FITC-labeled anti-mouse CD4 or PE-labeled anti-mouse CD8 (BioLegend, CA, USA) at 4 °C in the dark for 30 min. Samples were washed two times, then samples were fixed in PBS that contained 1% paraformaldehyde and were run on a Coulter Epics XL (Beckman Coulter) flow cytometer. The data analysis was performed using the FACS Diva software.

2.12. IFN-γ ELISPOT assays

At 30 days and 90 days post prime, numbers of IFN-γ-secreting splenocytes were analyzed by an approach similar to our previous work (Li et al., 2006a). Splenocytes (10^5) were plated in 96-well polyvinylidene fluoride (PVDF) membrane plates that had been coated previously with a capture antibody, in accordance with the manufacturer's protocol for the mouse IFN-γ precoated ELISPOT kit (Dakewe Biotech, Shenzhen, China). Stimulation of splenocytes was performed by the addition of 10 μL heat-inactivated VTT (1×10^6 pfu/mL) or phytohemagglutinin (PHA, 10 mg/mL) as a positive control, or RPMI 1640 alone as a negative control. Plates were incubated for 24 h, then plates were washed and developed using biotinylated antibody and streptavidin–horseradish peroxidase (HRP), and the number of spots was counted using a Biosys Bioreader.

2.13. Enzyme-linked immunosorbent assay

Quantification of secreted cytokines was performed using an enzyme-linked immunosorbent assay (ELISA) kit (GBD). Serum samples were obtained 30 days after the initial vaccination and used to detect mouse IL-2 and IL-4, by following the manufacturer's instructions. The absorbance at 450 nm of each sample was determined using an ELISA plate reader.

2.14. Measurement of IgG antibody against recombinant vaccinia virus vector

Antibody response was measured as described previously (Kan et al., 2012). Ninety-six well ELISA plates were coated overnight with crude vaccinia virus (2.5×10^5 pfu) in ELISA coating buffer (1×1 M Tris–HCl, pH 9.5) at 4 °C. Plates were washed and then blocked with 1% FBS/PBS at 37 °C for 1 h. Samples were washed three times, then serial fourfold dilutions of sera were reacted with vaccinia virus antigen that had been coated onto plates. Plates were incubated at 37 °C for 2 h and washed three times. HRP-conjugated goat anti-mouse IgG (Sigma) was added and incubated at 37 °C for 1 h. Plates were washed five times, and then the chromogen solutions A and B were added and plates were incubated in the dark at room temperature for about 30 min. The stop solution was added to terminate the reaction. The absorbance was read at 450 nm using a microplate reader. The cut-off value was calculated as follows: mean absorbance value of negative control +0.12.

2.15. Statistical analysis

Statistical analysis was performed using SigmaStat 3.5 (Systat Software) or SPSS. The statistical significance of differences was determined between groups using the two-way repeated measures analysis of variance (ANOVA) test or *t*-test. Survival analysis was performed using a log rank test. Statistical significance was expressed as $P < 0.05$.

3. Results

3.1. Construction of recombinant virus and virus morphogenesis in BHK-21 cells

The plasmid vector was constructed and inserted to the genome of recombinant vaccinia viruses as shown in Fig. 1. Virus was collected after cytopathic effect (CPE) appearing, plaques were isolated for expression of green fluorescent protein under a fluorescence microscope. Pure recombinant viruses were obtained after 10 rounds of plaque purification (Fig. 2). The genome of the recombinant vaccinia virus was extracted as the template to determine whether the TE3L gene had been removed from the genome of VTT via homologous recombination; polymerase chain reaction (PCR) was carried out with two pairs of specific primers as described in Materials and Methods. As shown in Fig. 2, two specific DNA bands of 573 bp (TE3L) and 720 bp (EGFP) were amplified as the positive controls, but no specific DNA bands were detected for

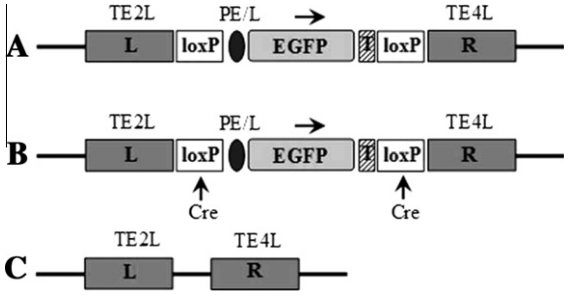

Fig. 1. Construction of plasmid vector (A) and genome structure of recombinant vaccinia virus (B) and gene-deleted virus (C). Enhanced green fluorescent protein (EGFP) (A) as a screening marker was inserted at the TE3L gene site of VTT and driven by the synthetic early/late promoter (PE/L) to obtain the recombinant virus (B) with deletion of TE3L gene but containing the EGFP gene. The recombinant virus TE3L⁻VTT (C) was generated through Cre/*loxP* site-specific recombination to remove the EGFP gene. T represents termination signal of vaccinia virus, *loxP* represents the *loxP* site, and Cre represents Cre recombinase.

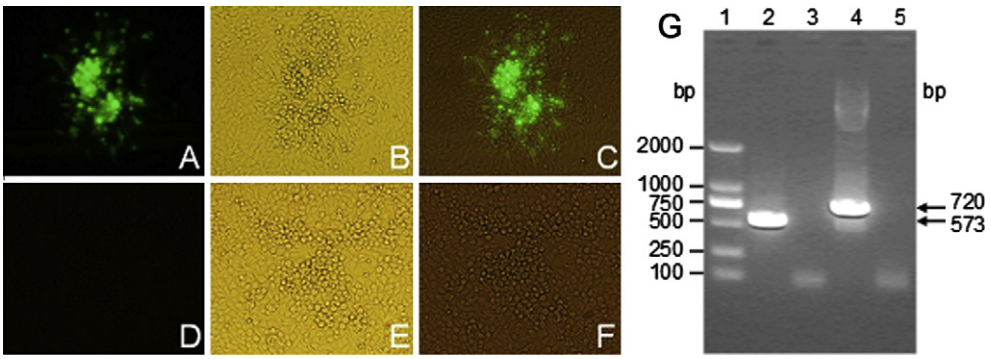

Fig. 2. Screening and identification of the recombinant virus. Screening of plaques in BHK-21 cells by fluorescence microscopy (magnification 200×, A–F). Expression of the fused enhanced green fluorescent protein (EGFP) observed in BHK-21 cell by fluorescence microscopy (A), or light microscopy (B), and image composition (C). TE3L⁻VTT–EGFP observed in BHK-21 cell by fluorescence microscopy (D), or light microscopy (E), and image composition (F). Analysis of the recombinant virus (TE3L⁻VTT) investigated by polymerase chain reaction (PCR) (G). Lane 1: DNA markers. Lane 2: positive control containing TE3L gene (573 bp). Lanes 3 and 5: PCR products of the genome of TE3L⁻VTT. Lane 4: positive control containing the EGFP gene (720 bp).

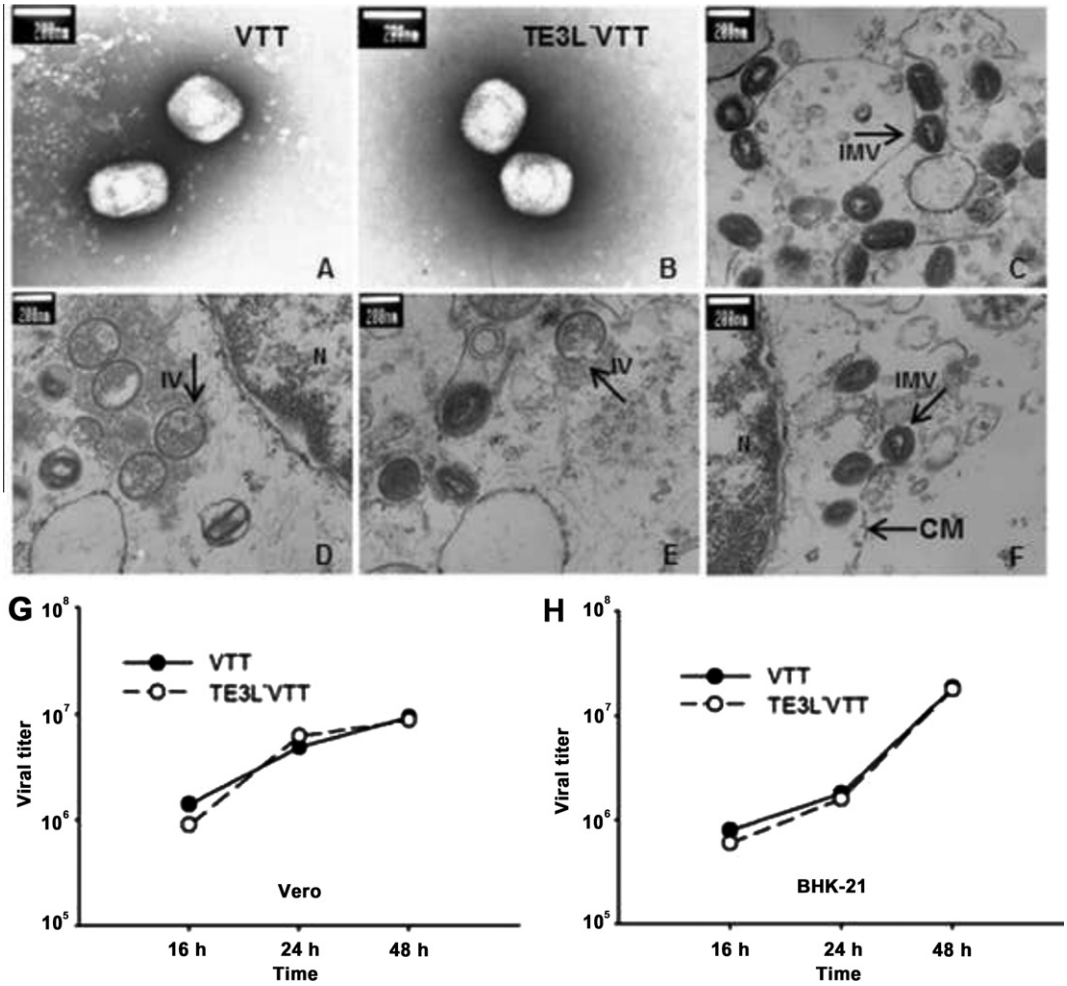

Fig. 3. Morphogenesis and replication of TE3L⁻VTT and VTT. (A) Electron micrographs of virus particles showing their regular shape: negatively stained electron micrograph of VTT. (B) Negatively stained electron micrograph of TE3L⁻VTT. (C–F) Morphogenesis of TE3L⁻VTT/VTT observed by transmission electron microscopy (TEM). Baby hamster kidney (BHK)-21 cells were infected with TE3L⁻VTT/VTT at 10 multiplicities of infection (MOI) and incubated for 24 h at 37 °C/5% CO_2 in air. The infected cells were scraped off the plate gently when the cytopathic effect (CPE) in the cells reached 70%. The fixed cells were then subjected to routine embedding and sectioning and then observed by TEM. Electron micrograph of VTT showed intracellular mature virus (IMV) (C), and immature virus (IV) (D). Electron micrograph of TE3L⁻VTT showed IMV (E), and IV (F). One-step growth curve of TE3L⁻VTT and VTT in BHK-21 cells (G), or Vero cells (H). Cells were infected with 5 MOI virus, and virus titers were measured after 16, 24, and 48 h post infection (pi).

the recombinant TE3L⁻VTT. In additional, the results of DNA sequencing of TE3L indicated that, the nucleotide sequence of TE3L gene from Tian Tan in our study is identical to that of TE3L and TE4L (GenBank: AF095689.1, reported by Jin Qi, et al.), the identity is 99.5%, and the homology of 99.3% is shown between the nucleotide sequence of TE3L gene sequenced here and that of E3L from WR strain, and the nucleotide number is consistent. The results suggested that the TE3L gene had been successfully

deleted and indicated that the mutants had been purified from the VTT parental virus. Morphological differences were observed by transmission electron microscope (TEM) and a one-step growth curve was performed to further confirm if removal of TE3L from an attenuated vaccine strain would affect virus morphogenesis and replication. Fig. 3 shows the results of TEM analysis after embedding, ultrathin sectioning and staining of cells infected with TE3L⁻VTT or VTT. As shown in Fig. 3A (VTT) and 3B (TE3L⁻VTT), both VTT and TE3L⁻VTT particles displayed regular morphologies and were brick- or ovoid-shaped, and the long axis of both was about 200 nm. As shown in Fig. 3C–D, there were two different forms of the wild-type VTT during the replication cycle. In Fig. 3E–F, TE3L⁻VTT intracellular mature virus (IMV) and the typical immature virus (IV) forms are shown by electron microscopy, while no marked differences between the shape and structure of TE3L⁻VTT and VTT were seen. And Fig. 3F shows virus particles of TE3L⁻VTT gather in the cell membrane, suggested that the virions was being released. And in Fig. 3G–H, there was similar growth kinetics in both TE3L⁻VTT and VTT in cell lines. The loss of TE3L had no apparent effect on normal virus replication and spread in BHK-21 and Vero cells. Together, these results indicated that the *TE3L* gene is not necessary for morphogenesis and normal replication of VACV in BHK-21.

3.2. Cell virulence of TE3L⁻VTT

The five cell types of BHK-21, Vero, PK15, MDCK and Hela were all permissive for the replication and infection of vaccinia virus Tian Tan (VTT) (Fang et al., 2005; Zhu et al., 2007). In this study, Vero, HeLa, MDCK, PK15 and BHK-21 cells were infected with TE3L⁻VTT or VTT to analyze cell virulence of VTT with the TE3L gene deletion. For BHK-21 cells infected with VTT, cell growth was suppressed by approximately 20%, 50% and 70% after 24, 48 and 72 h, respectively. As shown in Fig. 4, the viability of cells infected with VTT changed over time, and decreased gradually. By contrast, TE3L⁻VTT infection had little impact on the viability of PK15, MDCK and HeLa cells after 24–72 h. When BHK-21 cells were infected with TE3L⁻VTT, cell growth was suppressed by only about 10–20% after 48 and 72 h, respectively. As shown in Fig. 4, cell viability was shown to be significantly different for BHK-21 cells and MDCK cells infected with TE3L⁻VTT compared with those cells infected with VTT at 24 h ($P < 0.05$); more significant decreases in cell viability of VTT-infected cells were observed ($P < 0.01$) at 48 and 72 h. These results demonstrated that the absence of TE3L led to reduced cell virulence, which suggested that the recombinant virus would be a safer vector for vaccination than the wild-type VTT.

3.3. Virulence of attenuated vaccinia strain decreased in BALB/c mice

To evaluate the virulence of TE3L⁻VTT *in vivo*, we analyzed the body weight changes of BALB/c mice that had been infected with TE3L⁻VTT or VTT by intra-nasal inoculation. As shown in Fig. 5, TE3L⁻VTT administered at a range of doses did not cause weight loss in BALB/c mice during this observation period, and these infected mice also did not exhibit illness or death over a longer time period. Therefore, TE3L⁻VTT did not significantly affect the weight gain pattern of infected mice. By contrast, BALB/c mice that had been infected with VTT lost weight markedly within the first 10 days post-inoculation. Mice infected with 10^6 and 10^7 pfu of VTT reached maximal weight loss by 8–10 days after infection, and these values were significantly different between groups infected either with TE3L⁻VTT or the phosphate-buffered saline (PBS) control ($P < 0.05$). Therefore, the effect on body weight of the test mice correlated directly with the dose of virus inoculum. In addition, comparisons of TE3L⁻VTT-infected and VTT-infected

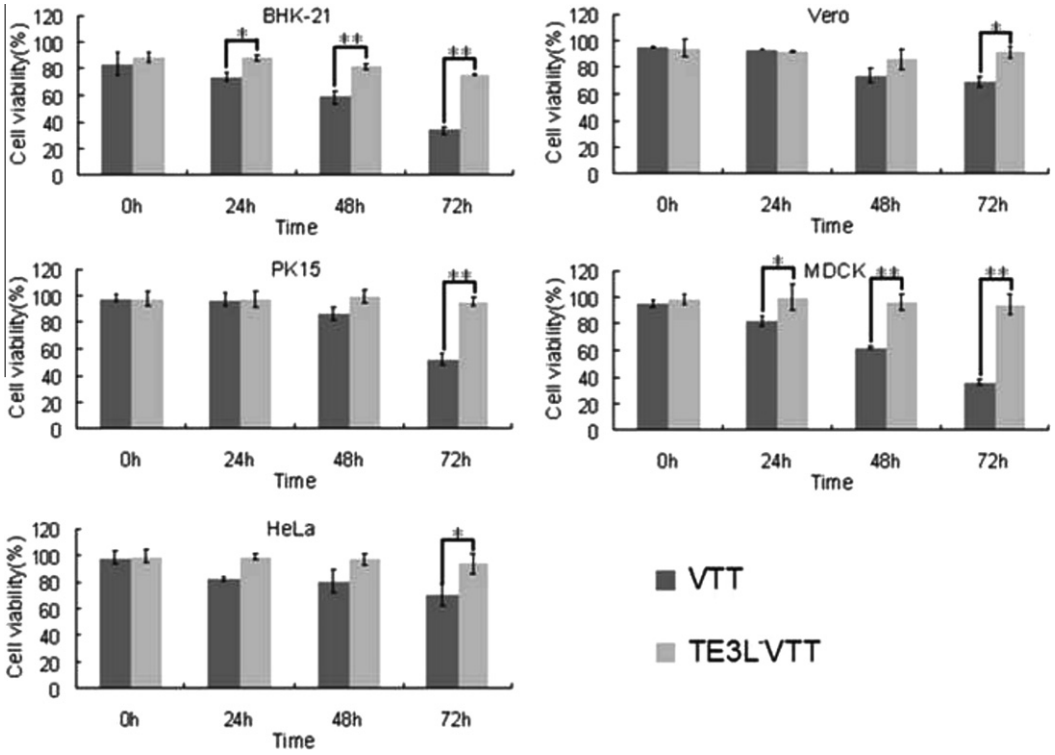

Fig. 4. MTT assay of cell viability after infection with TE3L⁻VTT or VTT. Baby hamster kidney (BHK)-21, Vero, PK15, Madin–Darby canine kidney (MDCK) and HeLa cells were seeded in 96-well plates (1×10^4 cells/well) 1 day before infection with 0.05 multiplicity of infection (MOI) of TE3L⁻VTT or VTT. Cell viability was measured every 12 h over a 72 h period, and data analysis was performed using independent-samples *t*-test for equality of means. When $P < 0.05$ (*) or $P < 0.01$ (**), the difference was considered to be significant.

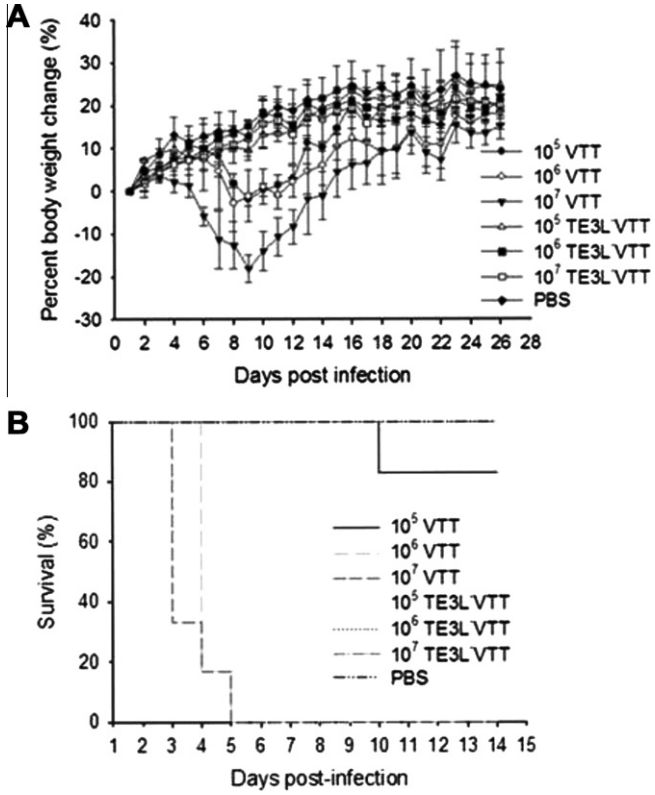

Fig. 5. Virulence analysis in BALB/c mice. Body weight changes of mice infected with TE3L⁻VTT or VTT by intra-nasal inoculation (A). Female BALB/c mice were infected intranasally with 10^5, 10^6 or 10^7 plaque forming units (pfu) of TE3L⁻VTT or VTT as a positive control and phosphate-buffered saline (PBS) as a negative control. Weight changes were monitored for 25 days after infection. Error bars indicate the standard error of the mean, and differences between groups were determined by two-way repeated measures analysis of variance (ANOVA). Comparison of survival of mice infected with TE3L⁻VTT or VTT by intracalvarial inoculation (B). Female BALB/c mice were infected by intracalvarial inoculation with 10^5, 10^6 or 10^7 pfu of TE3L⁻VTT or VTT as a positive control and PBS as a negative control. Deaths were monitored for 14 days post infection. Significant differences in the survival curves were analyzed using the SigmaStat long rank test.

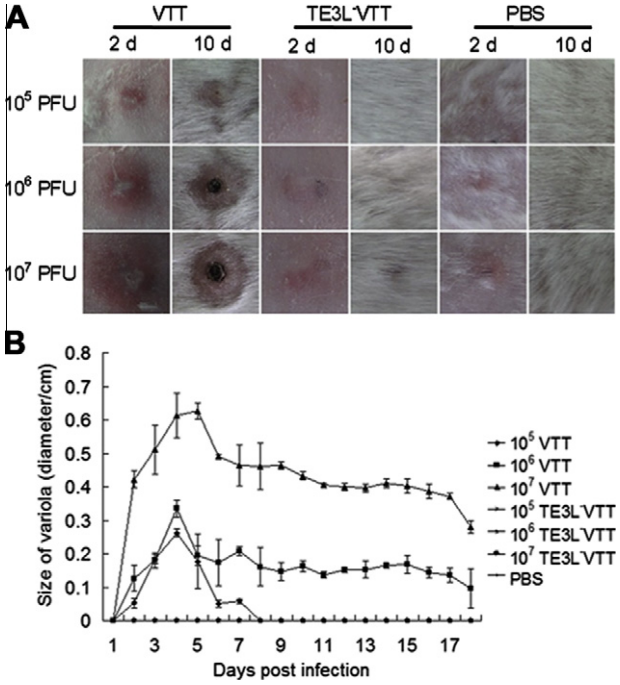

Fig. 6. Formation and resolution of lesions at the site of inoculation. Rabbits were infected with 10^5, 10^6 and 10^7 plaque forming units (pfu) of TE3L⁻VTT or VTT as a positive control and phosphate-buffed saline (PBS) as a negative control by intradermal injection. Pathological changes and the sizes of lesions at the sites of injection were monitored and recorded. (A) Rabbit skin infected with 10^5, 10^6 and 10^7 pfu of TE3L⁻VTT displayed light lesions by day 2, there were no visible scars by day 10 at the sites of infection. By contrast, rabbit skin infected with VTT displayed relatively severe lesions at the infection site with ulcerations. (B) Sizes of lesions at the site of infection were monitored over time. VTT infections caused the most severe lesions. The skin generally healed after infection, but the level of pathological changes was associated with the VTT dose used.

groups further showed that deletion of the *TE3L* gene attenuated the virulence of VTT *in vivo*. In conclusion, we found that complete deletion of the *TE3L* gene affected the virulence in mice. Because nerve cells or nervous system tissues are susceptible to VACV, it was necessary to test for neurovirulence to determine the true extent of the attenuation caused by the *TE3L* gene deletion. We determined the death rates of BALB/c mice that had been infected with either TE3L⁻VTT or VTT. Fig. 5 shows the percent survival of mice after intracranial infection over a 2-week period. The percent survival of mice infected with 10^5 pfu of VTT was 83% at 8 days after infection. The virus titer of VTT was $10^{7.5}$ $ICLD_{50}$/mL. No mortality was observed in mice infected with the TE3L mutant and given the high dose of 10^7 pfu. By day 5, survival rates of both the 10^6 and 10^7 pfu VTT groups differed from that of all other groups ($P < 0.05$). The results above suggested that deletion of the *TE3L* gene decreased the virulence and neurovirulence of the parental virus VTT. And the dose-dependent pattern was comparable to that of VTT and WR in mice published previously (Brandt and Jacobs, 2001; Fang et al., 2005).

3.4. No pock lesion in rabbits infected intradermally with TE3L⁻VTT

Administration of VACV to individuals intradermally can lead to pathological changes in the skin. Therefore, to test the severity of pathology of skin at the inoculation site, rabbits were infected with TE3L⁻VTT or VTT by intradermal injection. Rabbits infected intradermally with TE3L⁻VTT displayed only light lesions by day 2, and there were no visible scars by day 10 at the site of infection. In contrast, rabbits infected with VTT displayed comparatively severe lesions at the injection site, and resulting ulcerations required long times to heal (Fig. 6). Furthermore, no conspicuous ulcerations or indurations were observed at the inoculation sites of animals given TE3L⁻VTT over an 18-day period. These results suggested that TE3L⁻VTT was attenuated compared with VTT, and the presence of the *TE3L* gene contributed to the pathogenesis.

3.5. Cellular immune response to infection with TE3L⁻VTT

Cellular surface staining was performed to determine the percent of $CD4^+$ and $CD8^+$ splenocytes recruited in response to vaccination. As shown in Fig. 7, for $CD4^+$ splenocytes, the mean values of splenocytes from animals vaccinated with TE3L⁻VTT and VTT were both markedly higher than that of splenocytes from mock vaccinated mice ($P < 0.05$). The mean level of $CD4^+$ splenocytes from mice vaccinated with VTT was lower than splenocytes from TE3L⁻VTT, but there was no significant difference. The mean number of $CD8^+$ splenocytes from TE3L⁻VTT vaccinated group was higher compared with the mock vaccinated mice, but no significant difference. Overall, there were no marked differences between the levels of T cells from mice vaccinated with TE3L mutants or VTT. These results indicated that immunization could induce T-cell-specific responses and demonstrated that the removal of *TE3L* gene did not affect the magnitude and quality of the immune response compared with the parental strain. The induction of antigen-specific IFN-γ secretion was further evaluated in infected mice. ELISPOT

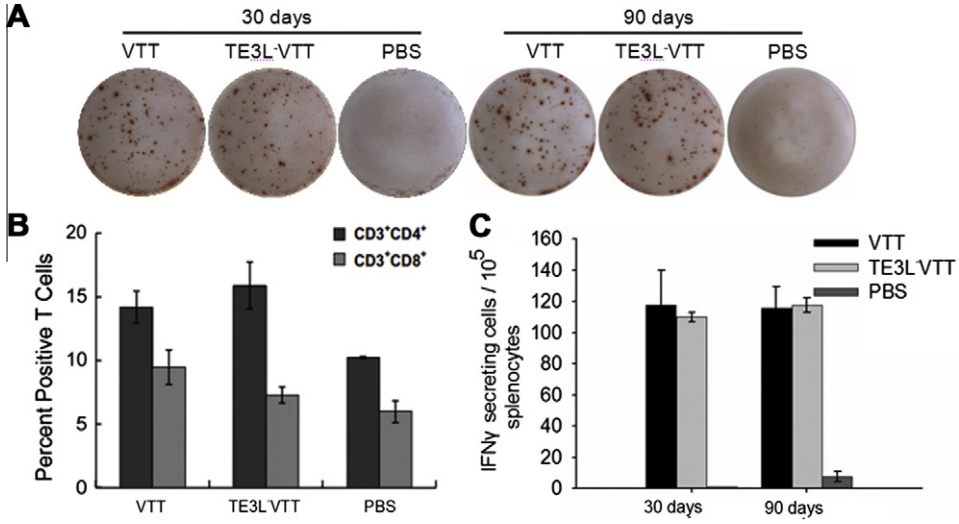

Fig. 7. Cellular immune response. (A) T cell subpopulations assay. On day 30 post prime vaccination, splenocytes from VTT and TE3L⁻VTT vaccinated mice were stained for different cell surface markers to enumerate percentage of T cell types. (B and C) Cytokine interferon (IFN)-γ secreted from splenocytes from vaccinated mice. Groups of BALB/c mice were immunized intramuscularly twice with 1×10^6 pfu TE3L⁻VTT or VTT. Mock-infected mice received PBS as negative control. Splenocytes were harvested 30, 90 days post prime and numbers of IFN-γ-secreting spleen cells were measured by ELISPOT assay.

assays were used to measure IFN-γ production as a result of stimulation with VTT antigens. As shown in Fig. 7, at 30 days and 90 days post prime, splenocytes from mice vaccinated with TE3L⁻-VTT produced significantly higher levels of IFN-γ than splenocytes from mock vaccinated animals ($P < 0.05$), however there was no significant difference in the number of IFN-γ-producing splenocytes between the TE3L⁻VTT and VTT groups. This result differs from previously reported data that found that there were more IFN-γ-producing spleen cells in mice infected with VACV TE3L mutants than in mice infected with the parental VACV (Jentarra et al., 2008). These results suggested that TE3L⁻VTT maintained high levels of immunological memory at 3 months post infection.

3.6. Humoral immune response to infection with TE3L⁻VTT

As previously reported, vaccinia virus-specific antibodies are considered to play a major role in protection against poxvirus infections (Isaacs, 2004). We measured the levels of anti-vaccinia virus antibodies in the sera of mice infected with TE3L⁻VTT or VTT to determine the effects of TE3L in VTT on antibody response. All mice had detectable levels of anti-vaccinia virus-specific IgG at 7, 14, 21, and 30 days after initial vaccination with TE3L⁻VTT or VTT virus (Fig. 8). Mice immunized with TE3L mutant virus or VTT produced significantly higher levels of antibodies compared with mice treated with PBS alone ($P < 0.05$). TE3L⁻VTT vaccinated mice developed markedly greater amounts of anti-vaccinia virus antibodies at 7 days after the first vaccination ($P < 0.05$) than mice infected with VTT. At days 14, 21, 30 post infection, antibody levels for TE3L⁻VTT vaccinated mice were not statistically different from the levels seen in VTT vaccinated mice. The production of cytokines IL-2, IL-4 was also compared in the sera of mice infected with TE3L⁻VTT or VTT viruses. Infected mice had statistically greater amounts of cytokines IL-2, IL-4 than the PBS-treated control mice, which indicated that an effective immune response was induced. As shown in Fig. 8, IL-2 or IL-4 levels for TE3L mutant vaccinated mice were not significantly different from the levels seen in VTT infected mice. This cytokine profile suggests that the TE3L protein in VTT does not affect the IL-2 or IL-4 secretion. In conclusion, inoculation with TE3L⁻VTT induced a humoral immune response that was stronger than inoculation with PBS.

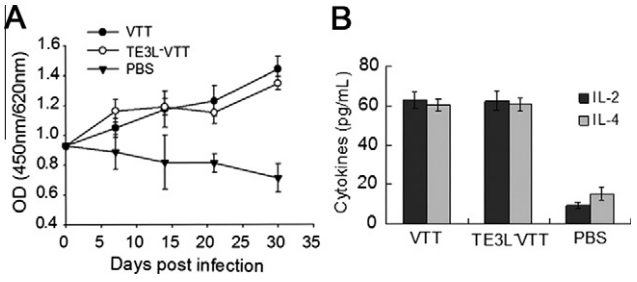

Fig. 8. Humoral response in vaccinated mice. (A) VTT-specific antibody response detected by ELISA. Blood samples were collected 0, 7, 14, 21 and 30 days after mice infected with 1×10^6 plaque forming units (pfu) of TE3L⁻VTT or VTT. On day 21 pi, mice received boost immunization were injected a second time through the same procedure. VTT-specific IgG were measured by enzyme-linked immunosorbent assay (ELISA) on VTT-coated plates and then read by microplate reader. (B) Serum cytokines in vaccinated mice detected by ELISA. Mice were infected intramuscularly twice with 1×10^6 pfu TE3L⁻VTT or VTT at weeks 0 and 3. Serum was collected on day 30 post the prime vaccination. Interleukin (IL)-2 and IL-4 productions performed by ELISA were demonstrated on the vertical axis (pg/mL).

4. Discussion

The E3L protein is synthesized early during the virus infection cycle and contains a C-terminal consensus dsRNA-binding domain and an N-terminal consensus Z-nucleic acid-binding domain (Chang et al., 1992). The E3L-encoded protein is a potent inhibitor of the antivirus activity of IFN-γ and is required for virulence in the mouse model. The deletion of E3L in VACV (Beattie et al., 1996; Langland and Jacobs, 2002) has been shown to cause replication deficiency in HeLa cells, while retaining full replicative capacity in BHK-21, CEF and RK13 cells. In the present study, we constructed a mutant of vaccinia virus Tian Tan (VTT) strain with the *TE3L* gene region deleted. The nucleotide sequence of TE3L gene from Tian Tan here is identical to that of TE3L and TE4L (GenBank: AF095689.1, reported by Jin Qi, et al.), but there is less one nucleotide "G" compared with TE3L and TE4L from GenBank: AF095689.1 (at position 339). And the nucleotide sequence of TE3L gene here is identical to E3L of the WR strain.

As deletion of *TE3L* has been shown to decrease virulence, the TE3L-deficient VTT virus was developed as a new replication-competent, attenuated vaccine vector to protect against heterologous diseases. We have previously evaluated the replication and

growth of the TE3L mutant, and found that the missing gene was not required for VTT replication. To further assess the attenuation of the recombinant virus, we also tested the viability of cells that had been infected with TE3L⁻VTT using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay. The growth of VTT and the mutant virus were similar in a panel of cell lines. The viability of cells infected VTT was significantly lower compared with that of cells infected TE3L mutant. This finding possibly demonstrated that deletion of TE3L reduced cytotoxic effects compared with wild-type VTT.

The influence of a virus on body weight gain is an important effect by which we can monitor virulence. Weight changes as a result of infection were analyzed; significant differences were found between mice that had been inoculated intranasally with TE3L⁻VTT or with VTT. In mice infected with VTT, the survival curve dipped to the minimum weight value between 8 and 10 days post-vaccination. Furthermore, the rate of weight change in mice infected with VTT was correlated with the virus dose. However, the weight changes of mice challenged with TE3L⁻VTT were coincident with that of control mice injected with PBS and weight slowly but consistently increased over time. A key aspect to evaluate virulence of TE3L⁻VTT is to determine its capacity to replicate in the nervous system *in vivo*, as VTT has shown a certain degree of neurovirulence (Buller et al., 1985) that can result in local or systemic pathological changes. BALB/c mice are sensitive to VACV and these mice are generally used to evaluate VACV virulence *in vivo* (Betakova et al., 2000). Intracutaneous inoculation in rabbits is also another method used to determine the pathogenicity of mutant VACV strains (Buller et al., 1988). Therefore, we selected both BALB/c mice and rabbits as experimental animal models to compare the virulence of TE3L⁻VTT and its wild-type strain. No death occurred among the mice challenged with the highest dose (10^7 pfu) of TE3L⁻VTT at 14 days post infection, nor during the following month. The data demonstrated that the neurovirulence of TE3L⁻⁻VTT was largely attenuated; this finding was consistent with other previously reported results (Izmailyan et al., 2006; Jentarra et al., 2008) and showed that the neurovirulence of TE3L-deleted VACV is reduced in the BALB/c mouse model tested. At 2 days' post-vaccination with the wild-type VTT, distinguishing features of VACV skin lesions were observed such as rubeosis and ulceration; ulcerations became encrusted at 10 days post infection. However, very mild skin changes were produced by the TE3L⁻VTT mutant, even at the highest virus dose used, and there were no marked pathological skin changes that resembled those mentioned above with VTT. The distinct differences at the inoculated skin sites between the mutant and wild-type viruses further supported the finding that virulence of the TE3L-deficient mutant was markedly reduced.

As previously reported, *TE3L* gene products blocked the induction of an antivirus state through IFN-regulated pathways (Langland and Jacobs, 2002; Rivas et al., 1998; Smith et al., 2001). We further tested vaccination efficacy and immunological characteristics in a mouse model to evaluate attenuation and efficacy of the attenuated TE3L-deficient vaccinia virus Tian Tan strain as a virus more appropriate for use in humans. CD4 and CD8 are receptors on the T-cell surface that have different functions. The level of CD4:CD8 expression is a key monitor used to analyze the immune state of an individual and in normal conditions this ratio is approximately 2:1. The mice inoculated with TE3L⁻VTT have a sustained immune system response. Stimulation of vaccinia-specific T cells in the spleen is associated with the secretion of Th1-type cytokines (IL-2, IFNγ) and Th2-type cytokines (IL-4). We observed that the T-lymphocyte responses were focused towards antigens in mice after TE3L⁻VTT vaccination. The number of IFN-γ-secreting T cells after immunization was monitored. At 90 days after initial vaccination, IFN-γ production was still maintained at high levels. This finding is consistent with the previous reports that $CD8^+$ T cells display memory cell markers 1 month after vaccination, and that these cells can be reactivated even at 6 months post-vaccination (Miller et al., 2008). As well as the cellular immune response, the humoral immune response also mediates protection against poxvirus infections (Belyakov et al., 2003; Chaudhri et al., 2006; Xu et al., 2004). We measured the effect of the TE3L mutation in VTT on the humoral immune response to infection in peripheral blood cells. Immunological regulation is coordinated by the combination of cytokines directed towards specific cells. The effect of the immune system is measured classically in mice by the levels of IL-2 and IL-4 produced. Both IL-2 and IL-4 productions were increased, with a substantial enhancement in TE3L⁻VTT mice compared with the PBS control mice. Overall, the removal of TE3L resulted in increased antibody production at 1 week post infection, which indicated that deletion of TE3L from TianTan strain vaccines does not affect their immunogenicity. We had previously noted that deletion of E3L results in increased induction of many pro-inflammatory proteins compared with the effects of other mutants (Langland et al., 2006). And in mice, the mutants of deletion or defective of E3L can protect against challenge with a high dose of wild-type vaccinia virus or heterologous poxvirus (Brandt et al., 2005; Denzler et al., 2011c), and the NYCBH vaccinia virus deleted for E3L can also protect rabbits against lethal challenge by rabbitpox virus (Denzler et al., 2011b). It is also reported that NYCBH vaccinia virus deleted for E3L gene showed partial protection against lethal monkeypox virus in cynomolgus macaques (Denzler et al., 2011a). So the basis for TE3L attenuated vaccinia virus Tian Tan strain changes needs to be characterized further. However, TE3L⁻VTT was shown to have a superior safety profile compared with the wild-type virus and has the potential to be developed into a virus-vectored vaccine for prevention or cure of diseases induced by different pathogens.

Acknowledgments

This work was supported by grants from the Genetically Modified Organisms Breeding Major Projects (2009ZX08006-002B), the Key Technologies R&D Programme of Jilin Province (10ZDGG007) and the National Natural Science Foundation of China (81101140).

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