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One time intranasal vaccination with a modified vaccinia Tiantan strain MVTTZCI protects animals against pathogenic viral challenge

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\section*{1. Introduction}

Smallpox is a serious and contagious human infectious disease with a mortality rate of up to 50\% [1,2]. The causative agent of smallpox is variola virus, a member of the orthopoxvirus genus. Since vaccination with VTT protected against variola virus, it was used for hundreds of millions of Chinese people to prevent smallpox infection between 1920 and 1980 (http://www.who.int/emc/diseases/smallpox/Smallpoxeradication.html). This led to the eradication of variola in China before 1980. Logically, VTT remains the first choice to be stockpiled for the country. However, the clinical safety of this vaccine has neither been carefully studied nor clearly documented. It was reported that VTT caused larger lesions after intradermal vaccination and was likely more virulent than other widely used smallpox vaccines such as Lister or Wyeth [13]. We recently demonstrated that VTT remains virulent in mice after intranasal inoculation, which restricts its use as a noninvasive smallpox vaccine [7,8].

During the smallpox eradication campaign, the most extensively used smallpox vaccine in China was the vaccinia virus Tiantan (VTT) strain [9–12]. VTT is also a member of the orthopoxvirus genus. Since vaccination with VTT protected against variola virus, it was used for hundreds of millions of Chinese people to prevent smallpox infection between 1920 and 1980 (http://www.who.int/emc/diseases/smallpox/Smallpoxeradication.html). This led to the eradication of variola in China before 1980. Logically, VTT remains the first choice to be stockpiled for the country. However, the clinical safety of this vaccine has neither been carefully studied nor clearly documented. It was reported that VTT caused larger lesions after intradermal vaccination and was likely more virulent than other widely used smallpox vaccines such as Lister or Wyeth [13]. We recently demonstrated that VTT remains virulent in mice after intranasal inoculation, which restricts its use as a noninvasive smallpox vaccine [7,8].

The parental VTT strain and cell lines have been described previously [14,15]. These cell lines were grown under conditions as described previously [14,15]. These cell lines were grown under conditions as described previously [14,15]. These cell lines were grown under conditions...
recommended by the American Type Culture Collection (ATCC, Rockville, MD, USA). The pathogenic vaccinia WR strain was purchased from ATCC (ATCC VR-1354) and propagated in Vero cells. Viral stocks were purified through a 36% sucrose cushion centrifugation. Virus for in vivo testing was further purified through sucrose density gradient centrifugation. The viral titer was determined by a traditional plaque-forming assay using crystal violet staining in Vero cells [14].

2.2. Construction of MVTTZCI

MVTTZCI was generated in Vero cells using a homologous recombination method [16]. Vero cells were infected with VTT and subsequently transfected with a shuttle vector ZCI containing a reporter green fluorescent protein (GFP) flanked with HA sequences. The homologous recombination also introduced an 84bp deletion to disrupt the HA gene. The recombinant virus was obtained by picking up GFP-positive plaque. Seven rounds of clonal purification were applied to generate MVTTZCI. For comparison purpose, VTT and MVTTZCI was propagated, purified and titrated in Vero cells in parallel.

2.3. Western blot analysis

Vero cells were infected with VTT or MVTTZCI at a multiplicity of infection (MOI) of 10. Cell lysates were generated 48 h.p.i. and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Western blotting was carried out with an anti-HA monoclonal antibody B2D10 (a generous gift of Dr H. Shida) and an anti-GFP polyclonal antibody (BD Biosciences, San Jose, CA, USA), respectively, as we previously described [16].

2.4. Analysis of VTT quasispecies

Vero cells were infected with 20 purified VTT clones at an MOI of 1. The infected cells were lysed by three freezing and thawing cycles. The cell lysates were treated with proteinase K at a final concentration of 50 ug/ml for 4 h and then cellular DNA was extracted by the conventional phenol–chloroform method. The PCR products were visualized by crystal violet staining and counted. IC50 or IC90 were determined by the highest dilution of mouse serum that generated 1% plaque reduction with 25% or 90% viral plaque reduction.

2.5. Viral replication in vitro and immunostaining of infected cells

Under multi-step growth conditions, cells were infected at a MOI of 0.05 in 100 μl of culture medium containing 3% fetal bovine serum (FBS). After 90 min of incubation at 37 °C, cells were washed three times with medium and replenished with fresh culture medium. Viral supernatant and infected cells were harvested at 0, 24, 48 and 72 h post-infection (p.i.). After freeze–thawing thrice, harvested samples were titrated in duplicate in Vero cells [16]. To determine the cell–to-cell spread of MVTTZCI, viral plaques were detected after immunostaining with a rabbit anti-VTT serum using a method previously described [15]. Briefly, target cells were grown to 90% confluence and then infected with 100 PFU MVTTZCI or VTT. After viral absorption for 90 min, cells were washed three times with culture medium and then incubated at 37 °C for additional 12 and 24 h for the detection of CPE [15].

2.6. The virulence of MVTTZCI in vivo

The inbred BALB/c mouse was chosen for the assessment of MVTTZCI virulence using a previously described method [17,18]. Groups of five-week old mice were inoculated intranasally with 0, 10^4, 10^5 or 10^6 PFU of MVTTZCI in 20 ul of PBS. The viral virulence was subsequently determined by the daily measurement of animal body weight change for a period of 10 days [19,20]. VTT was evaluated under the same conditions for comparison purpose. To evaluate the pathogenicity of MVTTZCI in immunodeficiency mice, groups of four SCID mice were infected intraperitoneally (i.p.) with 10^6 or 10^7 PFU of MVTTZCI, or with 10^3–10^6 PFU of VTT. Mice were weighted individually, and the averages were plotted. Mice that lose 25% of body weight were sacrificed according to the standard operating procedure of our animal facility. The number of animals that died of infection was also calculated. Uninfected mice were included as controls.

2.7. ICID50 measurement

Six groups of 3-week-old BALB/c mice (female and male half each) were inoculated intracranially with a series of diluted viruses of 10^2 to 10^6 PFU MVTTZCI in 10 μl of sterile PBS. The ICID50 value was determined on mice that succumbed between 1 and 14 days p.i. by calculating the 50% end point using the Reed–Muench method [21].

2.8. Replication kinetics of virus in mouse brain with or without immunodeficiency

Groups of 3-week-old BALB/c mice were administered with different doses of MVTTZCI or VTT via the intracranial route in 10 μl of PBS, respectively. Two mice in each group were sacrificed daily during the first 5 days p.i. for viral isolation. Infectious virions in the brains of inoculated mice were measured by culturing a serial diluted tissue homogenate in Vero cells. The viral titer was determined by counting plaque-forming units. Groups of 6-week-old SCID mice were injected intracranially with 10^6 or 10^4 PFU of MVTTZCI to access the replication profile of MVTTZCI in the brain of immunodeficiency mice.

2.9. Neutralization assay

To determine the serum neutralization against WR strain before challenge, a plaque reduction neutralization assay was used. Briefly, 100 PFU WR strain was mixed and incubated with serially diluted heat-inactivated mouse sera for 16 h at 37 °C. The mixture was transferred onto confluent monolayers of Vero cells in a 48-well plate and incubated for 90 min at 37 °C. On day 2, viral plaques were visualized by crystal violet staining and counted. IC50 or IC90 were determined by the highest dilution of mouse serum that generated 50% or 90% viral plaque reduction.
To understand the immunogenicity of MVTTZCI in vivo, we sought to delete the HA gene because it is related to the attenuation of various vaccinia viruses [17,23–25]. The new VTT variant named MVTTZCI was generated after a portion of the HA gene was deleted using a homologous recombination method [26–28]. To facilitate the purification of the MVTTZCI, we introduced the gene of green fluorescence protein (GFP) into the deletion site. After serial rounds of plaque clonal purification in Vero cells, a homogenous MVTTZCI stock was obtained as determined by the co-expression of GFP and vaccinia specific protein in infected cells. The expression of HA was detected in cells infected with VTT, but not with MVTTZCI using a HA-specific monoclonal antibody B2D10 (a generous gift from Dr. H. Shida) (Fig. 1). We also performed overlapping PCR screening analysis of MVTTZCI genome. In comparison to VTT, we found that MVTTZCI contains a large 11,944 bp genomic deletion from C2L to F3L in the left terminal region that we did not intend to make (Fig. 1). This deletion was further confirmed by sequence analysis. The vaccinia gene names are based on the sequence of vaccinia Copenhagen strain. The inset shows the results of Western blot analysis, which confirms the loss of the HA gene.

3. Results

3.1. Generation of the vaccinia MVTTZCI strain

To understand the origin of the 11,944 bp genomic deletion, we studied 20 randomly selected viral clones purified from VTT. We hypothesized that the smallpox vaccine VTT was probably consisted of a pool of variants namely quasispecies. We used a PCR-scanning method to analyze the VTT quasispecies. As shown in Fig. 2, we found that VTT genes are highly variable within the C2L to F3L region in the left terminal region. Nine out of the 20 clones contained various gene deletions. The results suggested that that MVTTZCI was likely derived from one of VTT variants containing the 11,944 bp genomic deletion. However, since none of these 20 clones contain the identical 11,944 bp deletion, we cannot exclude the possibility that our in vitro clonal selection process in Vero cells may also contributed to the emergence of MVTTZCI.

3.2. Reduced host cell range of MVTTZCI

Due to the significant loss of viral genomic fragments, we sought to determine the host cell range and replication capacity of MVTTZCI in vitro. Thirteen cell types, which have been previously used to determine the host cell range of VTT [14], were tested for MVTTZCI. We found that MVTTZCI replicates in mouse NIH3T3 cells in a semi-permissive way suggesting that its replication is not completely restricted in mouse cells (Table 1). It however displayed a rather limited replication capacity in three cell lines including RK13, MDCK, and C6, which were derived from rabbit, canine and rat, respectively (Table 1 and Fig. 3A). MVTTZCI did not seem to replicate in RK13 and MDCK cells at all even in the single round replication experiment with a MOI of 5. There was no viral particle formation in RK13 cells infected with MVTTZCI by electron microscopy analysis (data not shown). To further confirm this observation, we determined the level of viral spread among cells using an immunohistochemical staining method [15]. As shown in Fig. 3B, we found no cell-to-cell spread of MVTTZCI could be found in RK13 and MDCK cells. Since only singly infected cells were found up to 72 h (h) post-infection (p.i.), the data suggested that the cell-to-cell spread was likely absent in these two cell lines. Moreover, only a low level of viral spread was found in C6 cells with small clusters of infected cells identified 48–72 h p.i. (Fig. 3B). As controls, the replication and spread of MVTTZCI is indistinguishable to those of VTT in Vero cells, suggesting that the HA gene and the genes in the C2L-F3L region are non-essential for MVTTZCI replication and propagation in Vero cells (Table 1 and Fig. 3A and B).
3.3. In vivo virulence of MVTTZCI

The inbred BALB/c mouse was chosen for the assessment of MVTTZCI virulence using an intranasal (i.n.) inoculation model described previously by others [17,18]. As shown in Fig. 4A, none of the mice in either of the experimental groups died during the experimental period. However, in contrast to VTT, which caused a clear dose-dependent pattern of body weight loss in mice, those infected with MVTTZCI did not show signs of weight loss even in the 10^6 PFU group. These results demonstrate that MVTTZCI is attenuated with respect to its parent VTT.

To further investigate the neurovirulence of MVTTZCI, young BALB/c mice were infected via the intracranial (i.c.) route and the 50% lethal dose (ICLD50) determined using the Reed–Muench method [21]. The results of the ICLD50 are consistent with the intranasal infection model, MVTTZCI being a significantly attenuated virus (Fig. 4B). Mice neither developed signs of encephalitis nor were there any deaths during the 30 days of observation, even in the group given the highest dose (3.5 × 10^6 PFU/per mouse, data not shown). Since the ICLD50 of the parent VTT is 3.1 × 10^3 PFU, these results demonstrate that MVTTZCI is attenuated by at least 1000-fold, and is essentially non-neurovirulent.

3.4. MVTTZCI is safe in severe combined immunodeficiency disease (SCID) mice

To further determine the safety profile of MVTTZCI, we inoculated SCID mice with 10^6 and 10^7 PFU of MVTTZCI via the intraperitoneal (i.p.) route using a previously published method [29]. For controls, groups of mice received 10^3–10^6 PFU of the parental VTT or PBS. As shown in Fig. 4C, all animals that received 10^3–10^6 PFU of VTT died or were euthanized due to significant body weight loss (over 25%) according to the standard operating procedure. Fig. 4C shows that the average survival times of the VTT infected SCID mice are approximately dose-dependent with the animals eventually succumbing to as little as 10^3 PFU of VTT. In contrast, all of the mice that received 10^6 and 10^7 PFU of MVTTZCI survived and continued gaining their body weight during the experimental period. This 10,000-fold difference in dose represents a significant attenuated phenotype of MVTTZCI.

Table 1

| Cell line | ATCC code | Species | Organ | Morphology | Viral spread | CPE a,b | Viral replication c |
|-----------|-----------|---------|-------|------------|-------------|--------|--------------------|
|           |           |         |       |            |             | 12 h   | 24 h               |
| HeLa      | CCL-2     | Human   | Cervix| Epithelial | ++          | ++++   | 61.36 (P)          |
| MRC-5     | CCL-171   | Human   | Lung  | Fibroblast | +++         | ++++   | 52.50 (P)          |
| 293T      | CRL-11268 | Human   | Kidney| Epithelial | +++         | ++++   | 25.45 (P)          |
| WISH      | CCL-25    | Human   | Ammon | Epithelial | +++         | ++++   | 66.67 (P)          |
| RK13      | CCL-37    | Rabbit  | Kidney| Epithelial | *           | *      | 0.0031 (NP)        |
| MDCK      | CCL-34    | Canine  | Kidney| Epithelial | *           | *      | 0.035 (NP)         |
| CS4       | CCL-1-07  | Rat     | Brain; glial cell; glioma | Fibroblast | * | * | 3.83 (SP) |
| CHO-K1    | CCL-61    | Hamster, Chinese | Ovary | Epithelial | – | ++++ | 0.0292 (NP) |
| BHK-21    | CCL-10    | Hamster, Syrian | Kidney | Fibroblast | +++ | +++ | 5.69 (SP) |
| Vero      | CCL-81    | African green monkey | Kidney | Epithelial | +++ | ++++ | 275 (P) |
| COS-7     | CRL-1657  | African green monkey | Kidney | Fibroblast | +++ | ++++ | 89.58 (P) |
| CEF       | Primary   | Chick embryo | Assorted | Fibroblast | +++ | +++ | 112.5 (P) |
| NH133     | CRL-1638  | Mouse embryo | Embryo | Fibroblast | *** | +++ | 6.8 (SP) |

The biological properties of the parental VTT has been previously described (Fang et al. [14]).

a Virus spread as visualized by immunostaining after 72 h. –, no stained cells; +, foci of 1–4 stained cells; ++, foci of 5–25 stained cells; ++++, foci of >25 stained cells (Carroll and Moss, [15]).

b CPE was categorized by the following criteria: –, no difference from control; +, <25% CPE; ++, 25–50% CPE; ++++, >50–75% CPE; ++++ >75–100% or high level cell detachment.

c Virus replication (fold increase in virus titer) determined by dividing the virus yield at 72 h by the practical input titer. Cell lines were therefore categorized into permissive (P, >25-fold increase), semi-permissive (SP, 1-fold to 25-fold increase) and non-permissive (NP, <1-fold increase) cells.
3.5. Deficient replication of MVTTZCI in mouse brains

To further investigate the underlying mechanism that may account for the attenuated phenotype of MVTTZCI, we analyzed the replication kinetics of MVTTZCI in the brains of mice. A clear dose-dependent response was found in mice infected with the parental VTT (Fig. 5A), viral replication appearing to peak around 4 days p.i. Interestingly, MVTTZCI did not seem to replicate in the brains of the mice even when a dose of $10^6$ PFU was used and titers declined with time, which might suggest that the inoculated infectious MVTTZCI was somehow effectively cleared by the animals (Fig. 5B). Similar results were obtained when SCID mice were tested in an independent experiment (Fig. 5C).

3.6. Immunogenicity of MVTTZCI

To explore the potential of using MVTTZCI as a smallpox vaccine, we evaluated the immunogenicity of the virus in terms of inducing neutralizing antibodies against the pathogenic vaccinia WR strain. As shown in Table 2, one time i.n. immunization of MVTTZCI induced systemic neutralizing antibodies (Nabs) using the dose of $10^6$ PFU per mouse. The lower dose of $10^4$ PFU MVTTZCI, was not sufficient to elicit a detectable level of systemic Nabs via either route of inoculation. When compared with VTT in parallel (Table 2), a higher dose of MVTTZCI is apparently needed to achieve equivalent levels of systemic Nab response.

3.7. Protection of vaccinated mice against the pathogenic vaccinia WR strain challenge

To understand the in vivo efficacy of MVTTZCI, we challenged the vaccinated animals with a lethal challenge dose of $10^6$ PFU WR strain via the i.n. route 30 days after the single intramuscular (i.m.) vaccination [22]. The body weight change was monitored for 14 days after the viral challenge (Fig. 6). Unvaccinated mice began to lose their body weight three days post challenge and they all died after infection or were sacrificed due to 25% of total body weight loss. On the contrary, both MVTTZCI and VTT vaccinated animals that received the dose of $10^6$ PFU were completely protected (<3% of weight loss) (Fig. 6A). Moreover, for the lower dose groups ($10^4$ PFU), VTT vaccinated animals were completely protected while MVTTZCI also conferred significant protection (>8% of
**Fig. 4.** Virulence of MVTTZCI in mice after intranasal and intracranial inoculations. (A) Groups of five BALB/c mice (5-week-old) were inoculated intranasally with $10^6$, $10^5$ and $10^4$ PFU of MVTTZCI or VTT in 30 μl of PBS on day 0 (arrow), respectively. Mice inoculated with PBS served negative controls. The body weight changes were represented by the mean values of each group of mice p.i. over time. The error bar indicates the standard deviation (SD) of animals from each group. (B) Six mice per dilution group (3-week-old) were inoculated intracranially with 5-fold diluted MVTTZCI or VTT, respectively. The percentage of animals surviving was determined over 30 days observation p.i. None of mice died in MVTTZCI inoculated groups. (C) Groups of SCID mice (4 mice each group) were infected i.p. with MVTTZCI (10^6 and 10^7 PFU) or VTT (10^3, 10^4, 10^5 and 10^6 PFU). Mice inoculated with PBS were included as controls. The body weights of mice were measured individually and the mean values of each group were plotted. "†" indicates the loss of mice from infectious mortality or sacrifice due to 25% body weight loss.

**Fig. 5.** Replication kinetics of MVTTZCI in mouse brain. Groups of 10 BALB/c mice (3-week-old) were inoculated intracranially with the indicated doses of VTT (A) or MVTTZCI (B), respectively. Another group of 10 SCID mice were given MVTTZCI (C). Two mice in each group were sacrificed daily during the first 5 days p.i. The titer of virus in brain homogenates was determined in Vero cells using a plaque-forming assay. The error bar indicates the standard deviation (SD) of animals from each group.

**Fig. 6.** Protection of mice against pathogenic vaccinia WR strain challenge. Groups of five BALB/c mice (5-week-old) were immunized once with $10^6$ PFU (A) or $10^4$ PFU (B) of MVTTZCI or VTT strain via indicated routes, respectively. Mice received PBS were included as controls. Thirty days post-immunization, mice were challenged intranasally with a lethal dose ($10^6$ PFU, equivalent to over 100 LD<sub>50</sub>) of WR strain. Mice that lose 25% of body weight were sacrificed according to the standard operating procedure. The body weight changes were represented by the mean values of each group of mice p.i. over time. The error bar indicates the standard deviation (SD) of animals from each group.
weight loss). None of these MVTTZCI vaccinated mice died during the experimental period (Fig. 6B).

Since VTT is lethal via the i.n. vaccination, it was impossible to use it as a noninvasive mucosal vaccine. This option, however, is possible for MVTTZCI. Two groups of mice were vaccinated once through the i.n. route with 10^4 or 10^6 PFU of MVTTZCI. Thirty days after the single vaccination, animals were challenged with 10^6 PFU of the pathogenic WR strain via the same route. Fig. 6A and B show that MVTTZCI is equally effective in protection of mice against challenge with the WR strain following vaccination via the i.n. route when compared with the i.m. vaccination. Again, vaccinated animals that received 10^6 PFU i.n. were completely protected (<3% of weight loss), while mice that received the low dose of 10^6 PFU were also significantly protected (<8% of weight loss). None of these MVTTZCI vaccinated mice died during the experimental period.

4. Discussion

MVTTZCI is an attenuated variant of VTT. Moderate to severe adverse reactions are associated with smallpox vaccines currently stockpiled in several nations [6,30]. Recent studies, therefore, suggested that none or low virulent vaccinia strains should be selected for vaccination against smallpox [31]. In China, VTT is the licensed smallpox vaccine. Despite its application in smallpox eradication, whether or not VTT should be stockpiled to respond to a possible bioterrorist attack has attracted some debate. We previously demonstrated that VTT is less virulent when compared with the pathogenic WR strain [14]. VTT however remains neurovireulent and not feasible for noninvasive mucosal vaccination. Here, we report the generation of MVTTZCI, a variant of VTT. This variant remains replication-competent in several mammalian cells (Table 1) but does not cause body weight loss after intranasal inoculation or death after intracranial injection in mice (Fig. 4A and B). The significantly attenuated phenotype of MVTTZCI is also evident in immunodeficient SCID mice after a high dose of viral infection (Fig. 4C). Furthermore, we showed that the non-neurovireulent nature of MVTTZCI is likely due to the loss of the viral replication capacity in the brains of two mouse species (Fig. 5A and B). Therefore, the much improved safety profile of MVTTZCI has made it an attractive noninvasive smallpox vaccine candidate or a safe vaccine vector for other pathogens.

The attenuated phenotype of MVTTZCI is not solely determined by the HA gene. It has been previously demonstrated that the vaccinia genomic deletions may result in the generation of highly attenuated viral variants. For example, MVA contains six major genomic deletions which renders the virus replication incompetent in mammalian cells [32]. Similar situation applies to vaccinia strain NYVAC [25]. MVTTZCI is different from MVA and NYVAC in that it remains replication-competent in some mammalian cells tested (Table 1). HA is found on the plasma membrane of infected cells and the envelope of extracellular virus (EEV) but is absent from intracellular mature virus (IMV), the most abundant infectious forms of infectious virions [33,34]. Several studies showed that the loss of HA is associated with the reduced virulence of vaccinia viruses [17,23,35]. For example, MVA has the major deletion III in the HA promoter region [36]. The inactivation of HA gene was able to attenuate the neurovirulence of vaccinia WR strain by 10^4-fold as determined by the ICLD50 value but did not render the virus replication incompetent in mouse brains [23]. To our surprise, the removal of HA gene of VTT has resulted in the MVTTZCI with diminished neurovirulence (Figs. 4B, 5A and B). We speculated that other factors could have played a role. For this reason, we performed an overlapping PCR-scanning analysis of MVTTZCI genomic DNA to search for potential mutations especially corresponding to unstable deletion regions identified in MVA. We found that MVTTZCI contains a major 11,944 bp genomic deletion from HindIII fragment C2L to F3L in the left terminal region of viral genome that we did not intend to introduce. This deletion is located near the Delt site of MVA [32]. Besides HA, several genes in the deletion region could have contributed to the attenuated phenotype of MVTTZCI. First, it is known that N1L enhances virulence and replication of vaccinia virus in vivo [37]. Second, K1L is recognized as a host range gene (hr) that is essential to support the replication of vaccinia virus in RK13 cells [27,38,39], which are consistent to our finding that MVTTZCI becomes replication incompetent in RK13 cells (Fig. 3). Third, M2L is probably involved in the regulation of host NF-κB responses in virus infection which may potentially influence inflammation and the severity of vaccinia infection [40]. Fourth, K3L gene is an immunomodulatory and anti-apoptotic gene that inhibits IFN intracellular signaling pathway by preventing RNA-dependent protein kinase (PKR) activation [41–43]. Thus, deletion of K3L in vaccinia virus may render the virus sensitive to the antiviral effects of IFN and probably limit disease progression in vivo. Lastly, F1L is a mitochondrial-localized protein that functions to protect cells from apoptosis and inhibits cytochrome c release, which may affect viral propagation in vivo [44]. Therefore, the attenuated phenotype of MVTTZCI is likely due to the loss of HA as well as of several critical genes in the 11,944 bp deletion region. We found that the parental VTT consists of many viral variants (Fig. 2). We cannot exclude the possibility that other genes may also contribute to the attenuated phenotype of MVTTZCI.

MVTTZCI is an attractive noninvasive smallpox vaccine candidate or a safe vaccine vector for other pathogens for further development. Several studies have demonstrated that MVA and LC16m8 induced comparable protective immune response against pathogenic vaccinia virus infection in small animal models when compared with replicating vaccine strains [7,8,22,45,46]. A recent study also indicated that the longevity of protection induced by MVA is comparable to that induced by Lister strain [47]. Furthermore, vaccination with two doses of MVA was safe and well tolerated compared to the currently available smallpox vaccine Dryvax in humans. The vaccination also produced comparable cellular and humoral immune responses to one dose of Dryvax [47]. These attenuated vaccine candidates however are not available in China. Here, we demonstrated that MVTTZCI protects mice effectively from the challenge of a pathogenic vaccinia WR strain with an efficacy profile similar to that of VTT. Apparently, higher levels of neutralizing antibodies induced by either VTT or MVTTZCI contributed to the complete protection of animals against the lethal challenge of pathogenic WR strain. This finding should not be a surprise because major neutralizing determinants in B5R, H3L and other proteins were not affected by the deletions in MVTTZCI, which correlate with the protection of animals in previous studies [48,49]. Interestingly, although the low dose of MVTTZCI (10^4 PFU) did not induce detectable systemic neutralizing antibodies against WR strain, vaccinated animals were all significantly protected (Fig. 6B). We reason that the low dose of MVTTZCI had probably induced other protective immune mechanism effectively such as protective cell-mediated and mucosal immune responses, which will require further investigations. Since intranasal inoculation of MVTTZCI was equally effective compared to the intramuscular injection, our study has established proof-of-concept that the attenuated replicating MVTTZCI may serve as a safe and noninvasive smallpox vaccine candidate, which is a critical element for the mass vaccination program in developing countries including China, the nation with the world’s largest human population. A recent study indicated that aerosol immunization with non-replicating NYVAC and MVA vectored vaccines is safe, simple, and immunogenic [50]. It would be interesting to determine whether or not MVTTZCI will do better. Since a replicating MVTT-vectored vaccine induced higher level of neutralizing antibodies (∼100-fold...
against SARS-CoV infection via i.n. or i.o. (introrastral) inoculation when compared with the non-replicating MVA vector [51,52], the replication-competence likely offers greater advantage for improving the immunogenicity of MVTT-based vaccines. Moreover, the replication-competence may offer greater advantage for reducing the dose and frequency of smallpox vaccination. Further prime-boost studies should also be conducted to determine whether or not MVTTZCI vaccination prior to VTT or Dryvax would help to minimize the primary cutaneous lesion or other side effects related to the currently stockpiled vaccines. In addition, MVTTZCI is useful as a safe and replicating vector for the development of mucosal deliverable vaccines against infections of human immunodeficiency virus and avian influenza virus, etc. We have recently constructed an MVTTsVgpol to express simian immunodeficiency virus gag/pol/env using the MVTTZCI as a vaccine vector. We inoculated 10⁶ PUF of MVTTsVgpol into four rhesus macaques through intranasal inoculation, respectively. All four macaques tolerated the vaccination well without showing clinical signs of disease. There is therefore no evidence to indicate that the virulence of MVTTZCI is quite different in non-human primates when compared with mice. To proof the safety profile of MVTTZCI in humans, however, a careful clinical trial remains necessary. To this end, due to the replication-competency of MVTTZCI, it is necessary to carefully evaluate its use in immunocompromised non-human primate models and individuals if MVTTZCI-based vaccine will be used for immune therapy of AIDS patients. The GFP gene in MVTTZCI serves nicely as a negative selection marker for generating recombinant viruses and should not be included in vaccines developed for human use.

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