The Route of Inoculation Determines the Tissue Tropism of Modified Vaccinia Tiantan Expressing the Spike Glycoprotein of SARS-CoV in Mice

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The live replication-competent modified vaccinia virus Tiantan (MVTT) is an attractive vaccine vector, yet little is known about its tissue tropism and pathology in vivo. Recently, we demonstrated that a recombinant MVTT expressing the spike glycoprotein of SARS-CoV (namely MVTT-S) is superior to the non-replicating modified vaccinia Ankara (MVA-S) for inducing high level of neutralizing antibodies through mucosal vaccination. In this study, we further determined the tissue tropism and safety of MVTT-S after the vaccine was administrated through various routes including: intramuscular (i.m.), intranasal (i.n.), and intravaginal (i.vag.) inoculations, respectively. Using real-time PCR, nested PCR, immunohistochemistry and in situ hybridization assays, we found that MVTT-S was able to produce a transient infection in all cases within 48 hr post-inoculation, yet the major site of viral replication in various tissues or organs was dependent on the route of viral administration. We demonstrated that i.m. injection of MVTT-S primarily targeted draining inguinal lymph nodes, whereas mucosal inoculation had broader range of tissue infections. i.n. inoculation involved infections in lungs, kidneys, spleens and cervix lymph nodes while i.vag. administration targeted uteruses, ovaries, kidneys and spleens. Critically, the infection did not cause severe pathogenic consequences in infected tissues, which was consistent to the attenuated phenotype of MVTT-S. Our findings have implications for the optimization of vaccination route and for studies on the correlation between the magnitude of immune responses and the extent of tissue involvement in vivo. *J. Med. Virol. 82:727–734, 2010.* © 2010 Wiley-Liss, Inc.

INTRODUCTION

Mucosal surfaces contain the largest surface area of the human body and are the front line of natural defense against various pathogens. In fact, more than 80% of infectious disease pathogens in humans probably gain entry into the host through open mucosal membranes [Holmgren and Czerkinsky, 2005; Mestecky, 2005]. For example, human immunodeficiency virus type one (HIV-1), a mainly sexually transmitted virus, targets primarily the gastrointestinal and vaginal mucosa as entry sites for viral transmission and for virus replication and amplification [Lehner et al., 1991; Miller et al., 1993; Brenchley and Douek, 2008; Shacklett, 2008; Wu, 2008]. Since HIV-1 establishes its initial replication in mucosal tissues, the induction of sufficient mucosal immunity at the initial site of HIV-1 transmission becomes essential for a protective vaccine [O'Hagan, 1991; Forrest, 1992; Girard et al., 2008; Haynes and Shattock, 2008; Shattock et al., 2008; Broliden et al., 2009; Demberg and Robert-Guroff, 2009; Moldoveanu and Mestecky, 2009]. It is therefore believed that a successful vaccine should be able to elicit sufficient protective mucosal immunity against pathogens transmitted via corresponding mucosal routes.

To induce sufficient protective mucosal immunity, it is necessary to develop a proper antigen delivery vector through the mucosal route of inoculation. Various delivery vectors have been evaluated for this purpose [Davis et al., 1996; Caley et al., 1997; Fennelly et al., 1999; Pascual et al., 2001; Shata et al., 2001; Stevenson and Roberts, 2002; Osorio et al., 2003; Schlereth et al., 2003; Stolte-Leeb et al., 2008; Kastenmuller et al., 2009; Xu et al., 2009]. Among them, the poxviral vector is probably the most intensively studied live recombinant vector, and several studies have demonstrated its ability to induce mucosal immune responses against infectious pathogens [Gherardi et al., 2004; Wang et al., 2004; Kent et al., 2005]. In particular, vaccines based on the attenuated modified vaccinia virus Ankara (MVA) vector were effective in inducing protective responses against different respiratory viruses, such as SARS-CoV, influenza and respiratory syncytial virus, following immunization via mucosal routes [Sutter et al., 1994; Degano et al., 1999; Wyatt et al., 1999; Bisht et al., 2004; Olszewska et al., 2004; de Waal et al., 2004; Chen et al., 2005]. For example, intranasal inoculation of the MVA-based HIV-1 vaccine (named MVA-HIV) was immunogenic, whereas the intravaginal route was disappointing [Gherardi et al., 2004]. Since the mucosal immunogenicity of MVA-HIV is low, further studies are needed to improve the poxvirus vector system [Gherardi et al., 2004]. For this reason, MVA-HIV was co-delivered intranasally with the adjuvant cholera toxin (CT), which significantly enhanced the cellular and humoral immune response against HIV-1 antigens at mucosal surfaces of vaccinated mice. Due to the limitation of CT for human use, other vaccinia vector systems should be further optimized for stimulating mucosal immune responses.

Recently, we studied a newly developed vaccinia vector for mucosal vaccination. We demonstrated that the replication-competent modified vaccinia Tiantan (MVTT) expressing the spike (S) glycoprotein of SARS-CoV, namely MVTT-S, is superior to the non-replicating modified vaccinia Ankara (MVA-S) for inducing high level of neutralizing antibodies through mucosal vaccination [Huang et al., 2009]. It however remains elusive why the intranasal route of vaccination led to significantly stronger neutralizing antibody responses than the intramuscular injection. In this study, we determine the in vivo tropism and safety of MVTT-S after intramuscular, intranasal and intravaginal inoculations. We try to determine the extent of tissue involvement in supporting the vaccine efficacy. Our findings may have implications for the optimization of routes of vaccination and for studies on the correlation between the magnitude of immune response and the extent of tissue involvement in vivo.

MATERIALS AND METHODS
Virus and Animals

The recombinant vaccinia virus MVTT-S expressing GFP and SARS virus S protein has been described

previously [Chen et al., 2005; Zhu et al., 2007; Huang et al., 2009]. MVTT-S was grown and titrated in Vero cell line in DMEM supplemented with 10% fetal bovine serum (FBS). Specific pathogen-free female BABL/c mice of 6−8 weeks old were purchased from the Center of Disease Control in Hubei Province, China. The animal study protocol was reviewed and approved by the institutional animal care committee at the Wuhan University. Tissue homogenates derived from spleen, kidney, lung and uterus/ovary collected 8 and 12 hr post-three routes of inoculation were subjected to viral isolation using Vero cells. The replication of MVTT-S was tested in mouse NIH3T3 cells with a viral inoculum of 0.05 multiplicity of infection (MOI).

MVTT-S Inoculation

Groups of 6−8-week BALB/c mice were inoculated by intramuscular (i.m., 100 μl), intranasal (i.n., 20 μl), and intravaginal (i.vag., 20 μl) routes with MVTT-S, respectively. The same inoculum of 2.0E+07 PFU of MVTT-S was used for each vaccination route per animal. Post-vaccination with MVTT-S or placebo, 144 infected and 12 control animals were sacrificed at 4, 8, 12, 24, and 48 hr for evaluating acute infections and at 3, 6, 9, 12, 18, 28, and 42 day for studying chronic outcomes. Each group contained three animals.

Tissue Preparation

Tissue specimens including livers, kidneys, thymuses, hearts, lungs, spleens, cervix lymph nodes, inguinal lymph nodes, uteruses and ovaries were removed immediately after animals were sacrificed at the specified time points. For immunohistochemistry assay, tissues specimens were fixed by immersion in 4.0% formaldehyde−0.01 M sodium phosphate buffer (PBS, pH 7.2) for 24 hr and subsequently were embedded in paraffin. Serial tissue sections (5 μm) were mounted on resin-coated microscopic glass slides. For the in situ hybridization assay, tissues specimens were fixed by immersion in 4.0% paraformaldehyde−1.5%, 0.1 M sodium phosphate buffer (pH 7.2) and embedded in paraffin. Serial tissue sections (5−8 μm) were mounted on resin-coated microscopic glass slides. Genomic DNA was also isolated from kidneys, lungs, spleens, uteruses/ovaries using the standard protocol provided with the DNA extraction kit at 8 and 12 hr post-three routes of viral inoculation.

Real-Time PCR Assay

The whole blood samples were collected when mice were sacrificed. Serum samples were isolated from the whole blood after centrifugation at 4,000 rpm for 10 min. Poxviral DNA was extracted directly from viral particles concentrated from 150 μl serum using the DNA Blood Mini Kit (QIAGEN, Hilden, Germany). A 386 bp fragment of vaccinia virus F4L gene was amplified using a pair of PCR primers as follows: VACVF4L-F389-409 (5′-CGTTGG-AAAACGTGAGTCCGG-3′) and VACVF4L-

R774-754 (5′-ATTGGCGTTTTTTGCAGCCAG-3′). A control PF4L plasmid was constructed by using the PMD-18T vector (TaKaRa, Dalian, China) and inserting the 386-bp target F4L gene fragment. This plasmid was used to generate a standard curve so that the amount of viral DNA was properly quantified. SYBR GREEN was used for the real time PCR. The PCR program was: 95°C for 10 sec followed by 58°C for 20 sec for 30 cycles using the Bio-Rad Real-time PCR Cycler. The viral load was represented by the number of viral DNA copies in one milliliter of peripheral blood.

Immunohistochemical Assay

Tissue sections were first evaluated by Hematoxylin and Eosin staining. For the immunohistochemical assay, 5 µm thick tissue sections were deparaffinized and rehydrated through gradient xylene, alcohol and PBS. The sections were incubated with rabbit anti-MVTT-S serum at a dilution of 1/2,000, followed by the detection with the rabbit-SP staining kit (ZhongShan, Beijing) according to manufacturer's instruction.

In Situ Hybridization

For in situ hybridization, a 21-bp Digoxigenin-labeled probe of VAC-VF4L F389-409 (5′-CGTTGGAAAACGT-GAGTCCGG-3′) (Sun-Bio, Co., Shanghai, China) was used for the detection of vaccinia virus F4L gene in tissues. A nonreactive probe was used as a control. The functional activity of the probe was at 2 ng/µl. Tissue sections were incubated at 95°C for 10 min, and subsequently cooled on the ice for 1 min. The hybridization protocol was instructed in the manual of in situ hybridization Kit (Boster Bio, Beijing, China). The localization of positive cells was captured with the CCD microscope camera at the magnification of 400×.

RESULTS

Infection of BALB/c Mice by MVTT-S

Blood samples were taken from BALB/c mice experimentally inoculated with MVTT-S through intramus-

cular (i.m.), intranasal (i.n.), and intravaginal (i.vag.) inoculations, respectively. An equal dose of 2.0E+07 PFU of MVTT-S was given to each animal. The serum viral load was determined by real-time PCR assay. As shown in Table I, we found that all animals had detectable viral DNA regardless of the route of vaccination from 8 to 48 hr post-infection. The average level of viral load ranged between 5.57E+02 to 2.62E+03 DNA copies/ml blood during the acute phase of infection. The i.m. injection led to about 10-fold higher viral load when compared with the i.n. and i.vag. inoculations. Consistently, we found that there was an increasing trend, with a peak around 12 hr post-viral inoculation, of infected peripheral blood mononuclear cells (PBMCs) by FACS analysis (Fig. 1). On average, i.m. injection also led to more infected mononuclear cells. The infection, however, appeared to be transient in all animals because no viral DNA was detected 72 hr post-vaccination (Table I). Similarly, the number of infected PBMCs also dropped significantly 24 hr post-viral inoculation (Fig. 1). We also found that MVTT-S was non-permissive and did not induce plaque formation in mouse NIH3T3 cells when compared with parental VTT (data not shown). Only individual cells were infected as indicated by GFP expression. All animals appeared to be clinically healthy without evident body weight loss or signs of sickness. To further address the safety issue, we inoculated four Chinese macaques with MVTT-S at a dose of 1.0E+08 PFU through the intranasal route. All four macaques have not shown any signs of clinical sickness in over 4 months of observation.

i.m. Inoculation Did Not Result in Detectable MVTT-S Replication in Mucosal Tissues

Despite the higher levels of viral load detected among BALB/c mice who received the i.m. injection of MVTT-S, the tissue distribution of the virus was rather limited during the course of infection. Using the immunohistochemistry assay, viral antigen was primarily detected in draining inguinal lymph nodes in three of the six mice around 8 and 12 hr post-inoculation (Table II and Fig. 2). No detectable MVTT-S antigen was found by IHC in

TABLE I. Average Viral DNA Load in Mice Infected With MVTT-S by Various Routes of Inoculation

Group	Systemic inoculation i.m.	Mucosal inoculation i.n.	i.vag.
4 (hr)	<100 (copies/ml)	<100	<100
8	<100	<100	5.91E+02 (1.78E+01)
12	2.62E+03 (1.14E+02)	7.43E+02 (9.62E+01)	9.27E+02 (4.67E+01)
24	2.31E+03 (9.54E+01)	5.57E+02 (1.12E+01)	6.S5E+02 (8.20E+01)
48	1.47E+03 (9.90E+01)	<100	7.39E+02 (1.28E+02)
72	<100	<100	<100
144	<100	<100	<100
216	<100	<100	<100
288	<100	<100	<100
432	<100	<100	<100
672	<100	<100	<100
1,008	<100	<100	<100

hr, hour; i.n., intranasal; i.m., intramuscular, i.vag., intravaginal. The average viral load is represented by DNA copies/ml blood. The number in parentheses represent standard deviation.
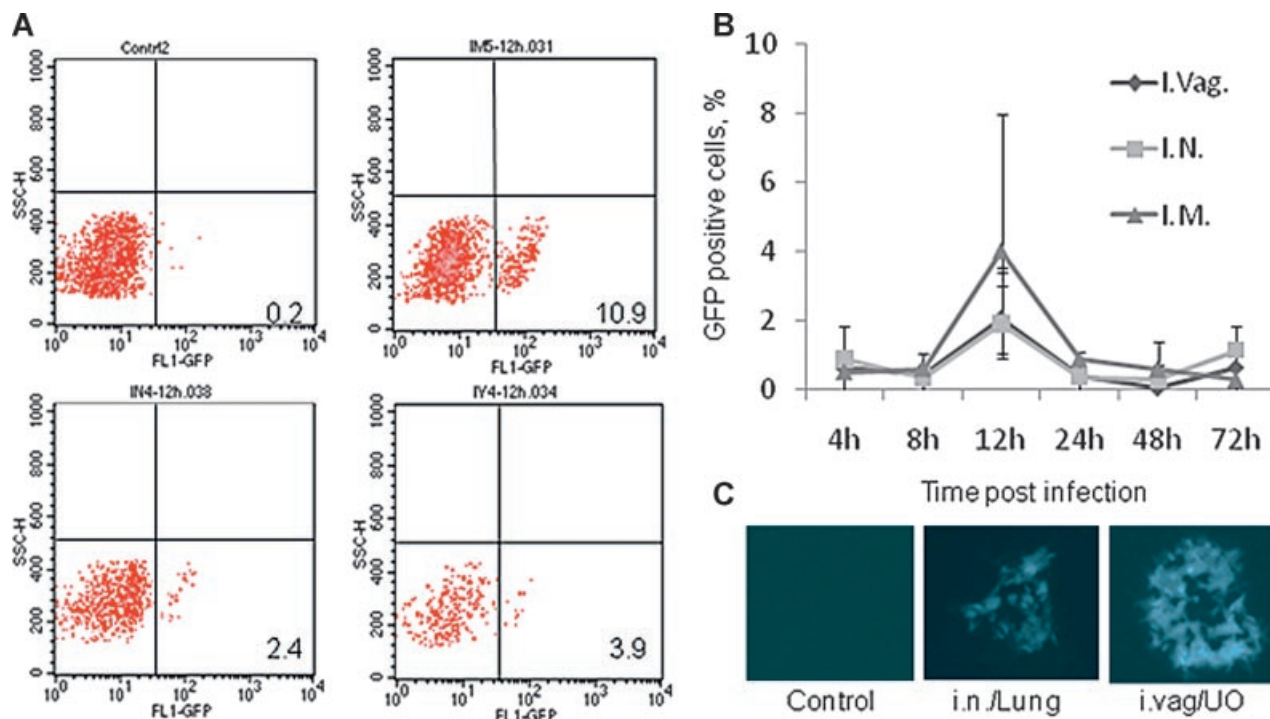
Fig. 1. Detection of MVTT-S infected cells in peripheral blood (**A,B**) and tissues (**C**) after intramuscular (i.m.), intranasal (i.n.), and intravaginal (i.vag.) inoculations, respectively. The percentage of vaccinia-infected cells was determined by FACS analysis for GFP expression at 12 hr (A) or overtime (B) post-inoculation (p.i.). Six mice were included for each route of inoculation. The average values are plotted in B with standard error bars indicated. C: Infectious viruses isolated from lung (8 h, p.i., i.n.) and uterus/ovary (8 h, p.i., i.vag.) formed typical plaques in Vero cell monolayer.

other tissues including the spleen, thymus, liver, kidney, lung, heart, uterus and ovary (data not shown for thymus and heart). In addition, viral DNA was detected in a kidney (1/3, 12 hr) but not from spleen, lung and uterus/ovary extracts by nested PCR analysis. Live MVTT-S, however, was not isolated from these tissue compartments 8 or 12 hr post-viral inoculation.

i.n. Inoculation Primarily Targeted Lung and Led to Broad Range of Tissue Infections

After the i.n. inoculation of 2.0E+07 PFU MVTT-S per mouse, the viral antigen was not only found in the cervical lymph nodes but also in the lungs, spleens and kidneys (Table II, Figs. 2 and 3). In particular, extensive infections were found in the lungs of all three mice tested at 8 hr post-infection. In the lung of the presenting mouse, most pneumocytes expressed vaccinia-specific antigens (Fig. 3). Moreover, viral antigens were found in the lymph nodes of all three mice tested at 24 hr p.i., in the kidneys of three of the six mice at 12–24 hr p.i. and in the spleen of one of the three mice at 8 hr p.i. The endothelial cells of cortical tubules were mainly infected in the kidneys (Fig. 3). Interestingly, the infected animals seemed to resolve the infections quickly. Infections in the targeted tissues were apparently

TABLE II. Detection of MVTT-S Antigens in Different Tissues After Various Routes of Inoculation

Tissue	i.m.					i.n.					i.vag.				
	4 (hr)	8	12	24	48	4	8	12	24	48	4	8	12	24	48
Liver	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Kidney	—	—	—	—	—	—	—	2/3	1/3	—	—	—	—	1/3	2/3
Thymus	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Heart	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Lung	—	—	—	—	—	—	3/3	—	—	—	—	—	—	—	—
Spleen	—	—	—	—	—	—	1/3	—	—	—	—	—	1/3	2/3	2/3
Lymph node	—	2/3	1/3	—	—	—	—	1/3	3/3	2/3	—	—	—	—	—
Uterus	—	—	—	—	—	—	—	—	—	—	2/3	3/3	—	—	—
Ovary	—	—	—	—	—	—	—	—	—	—	—	—	3/3	—	—

Data are representative of three independent experiments.
hr, hour; i.n., intranasal inoculation; i.m., intramuscular inoculation; i.vag., intavaginal inoculation.
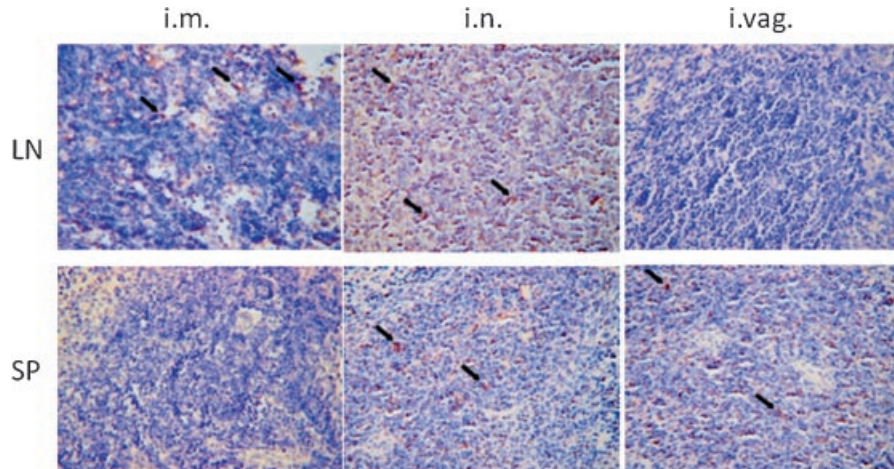
Fig. 2. Infection of lymph tissues by MVTT-S after various routes of inoculations in mice. MVTT-S positive cells are identified in lymph nodes (LN) and spleens (SP) after intramuscular (i.m.), intranasal (i.n.), and intravaginal (i.vag.) inoculations, respectively. The vaccinia-specific cells are indicated by arrows after the immunohistochemistry staining using a rabbit anti-MVTT serum. Magnifications 400×.

transient because the virus was only detected between 8 and 48 hr p.i. Again, despite the finding of many infected cells in the lungs, spleens, lymph nodes and kidneys, no significant pathogenic changes were found in these tissues (Figs. 2 and 3). For example, there was no sign of lung damage despite detecting significant amount of viral antigens there (Fig. 3). Viral antigens, however, were not detected in the other tissues tested including thymus, liver, heart, uterus and ovary (Fig. 3 or data not shown). Viral DNA was detected in lungs (3/3, 8 h), spleens (1/3, 12 h), and kidneys (1/3, 8 h) but not from uterus/ovary extracts by nested PCR analysis. Live MVTT-S was also isolated from infected lungs 8 hr post-viral inoculation (Fig. 1C).

i.vag. Inoculation Primarily Targeted the Reproductive Tissues

In contrast to i.m. and i.n. inoculations, i.vag. route targeted the reproductive organs predominantly. As shown in Figure 3, large amount of infected cells was found in the uteruses and ovaries in addition to some infected cells in the spleens and kidneys. Both endothelial cells of invaginations in the uterus and granulose cells in the ovaries were infected. The early detection of MVTT-S infected cells in the uteruses of five of the six mice at 4–8 hr p.i. is particularly unique to the i.vag. route of viral inoculation. Moreover, extensive infections were found in the ovaries of all three mice tested at 12 hr p.i. Viral antigens were also found in the spleen of 5 of the 9 mice tested at 12–48 hr p.i., and in the kidneys of three of six the mice at 24–48 hr p.i. Of note, significant level of MVTT-S infection in the ovaries was only transiently found in these three mice at 12 hr p.i. Again, the transient infection of MVTT-S did not seem to lead to significant pathology in the spleens, kidneys, uteruses and ovaries. MVTT-S infected cells were found in neither inguinal nor cervix lymph nodes. Viral DNA

was detected in lung (2/3, 8 h), spleen (2/3, 8 and 12 hr), and kidney (2/3, 8 and 12 hr) as well as uterus/ovary (3/3, 8 and 12 hr) extracts by nested PCR analysis. Live MVTT-S was mainly isolated from infected uterus/ovary 8 and 12 hr post-viral inoculation (Fig. 1C). Unexpectedly, MVTT-S antigen was found in two thirds of mice on day 6, 9, and 12 post-i.vag. inoculation, likely suggesting a longer period of viral persistence in the ovaries.

In Situ Hybridization of MVTT-S Infected Cells

To further confirm the distribution of MVTT-S infected cells, tissue samples were further tested by in situ detection of vaccinia viral DNA. A digoxigenin-labeled VACVF4 probe was designed for the detection of vaccinia F4L gene, which is intact in MVTT-S. We found that the distribution of positive cells was consistent to that of immunohistochemistry staining. As shown in Figure 4, positive cells were readily detected in tissues which showed viral antigen expression. The positive cell types are consistent to the findings of immunohistochemical staining including pneumocytes in the lungs, endothelial cells of cortical tubules in the kidneys, endothelial cells of invaginations in the uteruses and granulose cells in the ovaries (Fig. 4).

DISCUSSION

Previous studies have demonstrated that the level of anti-S neutralizing antibodies correlated with the protection against pathogenic SARS-CoV [Bisht et al., 2004]. We and others have also demonstrated that MVA-S, which is less immunogenic than MVTT-S, induced sufficient protection against pathogenic SARS-CoV in both mouse and monkey models [Bisht et al., 2004; Chen et al., 2005; Huang et al., 2009]. To further investigate the mucosal vaccinology, the primary goal of the study was to study the tissue tropism and safety of MVTT-S
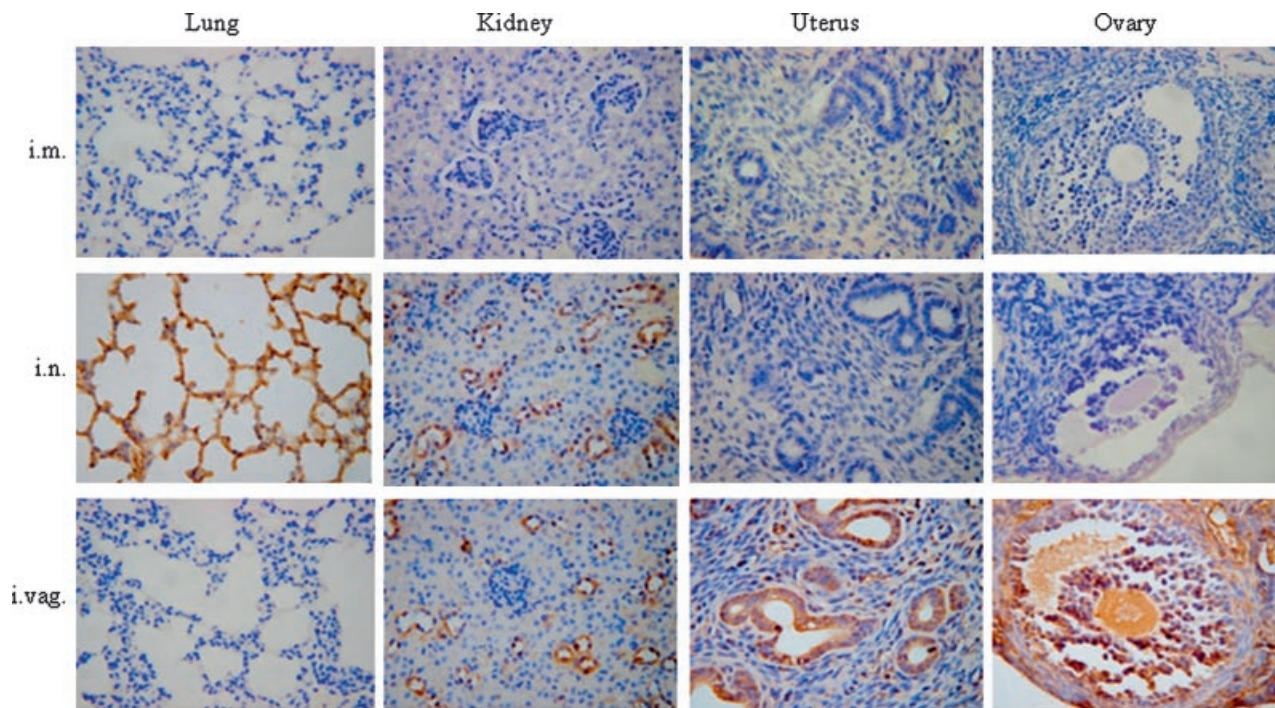
Fig. 3. Tissue tropism of MVTT-S after various routes of inoculations in mice. MVTT-S positive cells are identified in lungs, kidneys, uteruses and ovaries after i.m., i.n., and i.vag. inoculations, respectively. The vaccinia-specific cells are indicated by arrows after the immunohistochemistry staining using a rabbit anti-MVTT serum. Magnifications 400×.

after the vaccine was administrated through various routes. In this study, by collecting data from 156 mice, we studied the in vivo tropism of MVTT-S to understand the optimal route of vaccination and the correlation between the magnitude of immune responses and the extent of tissue involvement in vivo. Our previous study demonstrated that much higher levels of neutralizing antibodies were induced through i.n. inoculation than through i.m. and i.vag. routes in mice. Here, we provide evidence that the tissue distribution of MVTT-S is quite different when a distinct route of vaccination was applied, which also to some extent explains the immunogenicity outcomes observed.

MVTT-S appears to be a well-tolerated vaccine without any signs of causing clinical sickness and tissue pathology in animals. In comparison to our previous

studies, a 20-fold higher dose of MVTT-S (2.0E+07 PFU vs. 1.0E+06 PFU) was used in this study trying to achieve robust viral infections in vivo [Huang et al., 2009]. All animals appeared to have had transient infections as determined by measurements of viral DNA load and the number of infected peripheral cells (Table I and Fig. 1). The higher level of viral DNA copies detected from i.m. infected mice than animals infected through mucosal routes is not a surprise because it could be accounted for by better access to the circulatory system and diffusion alone. Despite the detected viral DNA and infected cells, all experimental mice were clinically healthy without any signs of sickness including no reduction in body weight when compared with placebo animals. These findings are in fact consistent to our previous data that MVTT-S is a significant attenuated
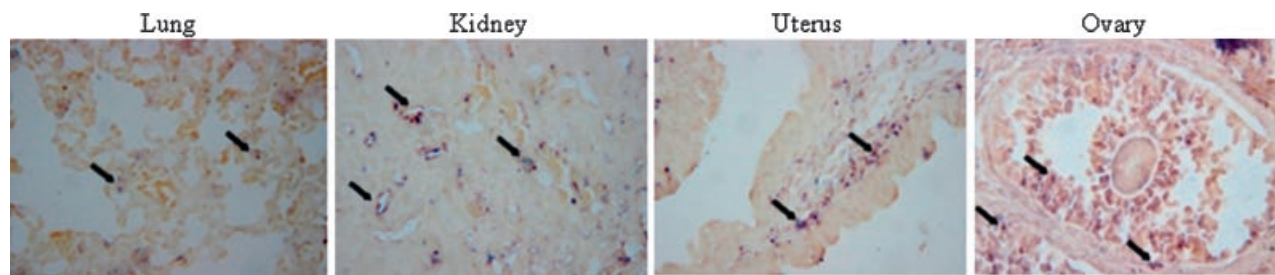

Fig. 4. The replication of MVTT-S in various tissues as determined by the ISH assay. MVTT-S positive cells, as indicated by arrows, are identified in lungs, kidneys, uteruses, and ovaries after i.m., i.n., and i.vag. inoculations, respectively. Magnifications 400×.

strain of vaccine [Huang et al., 2009]. Moreover, by evaluating the pathogenic effects of MVTT-S in all tissue sections overtime, we demonstrated that the vaccine strain did not cause any severe pathological changes in organs even with significant viral infections (Figs. 2 and 3). Similar conclusions were obtained when HE stained tissue sections were evaluated by our veterinary pathologists. These results are likely related to the restricted replication capacity of MVTT-S in mouse NIH3T3 cells. Since mouse is genetically distant from humans, our data cannot preclude the possibility that MVTT-S is more virulent or pathogenic in primates. To address this issue, four Chinese macaques inoculated with MVTT-S at a dose of 1.0E+08 PFU through the intranasal route have not shown any signs of clinical sickness in over 4 months of observation. We are therefore convinced that MVTT-S is a safe and well tolerant SARS vaccine candidate. Despite our findings, the safety profile of MVTT-S in humans still requires careful clinical studies in the future.

The tissue tropism of MVTT-S was not the same despite inducing similar levels of transient infection through three different routes of vaccination. Ramirez et al. previously demonstrated that i.n. inoculation of MVA targeted the nasal associated lymphoid tissue and the lungs, whereas i.m. inoculation led to distribution of MVA in almost all lymphoid organs, lungs and ovaries [Ramirez et al., 2003]. In our study, although i.m. injection of MVTT-S resulted in relatively higher viral DNA loads (Table I), the range of infected tissues appeared to be limited when compared with i.n. and i.vag. inoculations as determined by both IHC and nested PCR assays. Unlike MVA, after i.m. injection, a significant amount of MVTT-S antigens was only found in the draining lymph nodes (Fig. 2) whereas few infected cells were found in other tissues. In contrast, viral antigens were found in the lungs, lymph nodes, spleens and kidneys after i.n. inoculation and in the spleens, kidneys, uteruses and ovaries after i.vag. administration. MVTT-S was also isolated from lungs after i.n. or from uterus/ovary homogenates after i.vag. inoculations, respectively. The mucosal delivery of MVTT-S, either i.n. or i.vag., clearly infected a broader range of tissues and organs. Since the data are based on multiple animals and tissue specimens collected at multiple time points post-infection, it is very likely that the route of viral inoculation determined the in vivo tropism of MVTT-S. Our data also provided evidence to show that it is possible that different vaccinia viral vectors (MVTT vs. MVA) may have distinct profiles of in vivo tropism.

Our findings may have implications for the optimization of vaccination route and for studies on the correlation between the magnitude of immune responses and the extent of tissue involvement in vivo. We previously demonstrated that i.n. inoculation of MVTT-S is superior to i.m. for inducing higher levels of neutralizing antibodies against SARS-CoV [Huang et al., 2009]. Here, we provide evidence that the i.n. route of vaccination led to a broader range of tissue involvement including: lung, lymph node, spleen and kidney when compared with the i.m. injection. Interestingly, although the focal infection appeared to be transient, the progressive tissue involvement especially in the respiratory system likely contributed to significant immune stimulation. A possible explanation is probably related to the fact that a large number of antigen presenting cells reside in the lungs and its associated lymph tissues [Sertl et al., 1986]. However, how these antigen presenting cells lining the mucosal tissues in the respiratory system would have played a critical role in immune stimulation still require further investigation. Although i.vag. inoculation also resulted in a broader range of tissue infections in the spleens, kidneys, uteruses and ovaries, these tissues are not the primary site for inducing strong immune responses, except for the spleen. In addition, although MVTT-S maintains the replication-competent property in mammalian cells, the time of viral persistence appeared to be rather short in the tissues examined. Only the ovaries contained detectable viruses 48 hr post-i.vag. inoculation. If i.vag does not show any advantage for inducing better immune responses, this route of vaccination can be avoided. Besides, the long-term consequences of infections in the ovaries and uteruses remain unknown in humans.

ACKNOWLEDGMENTS

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