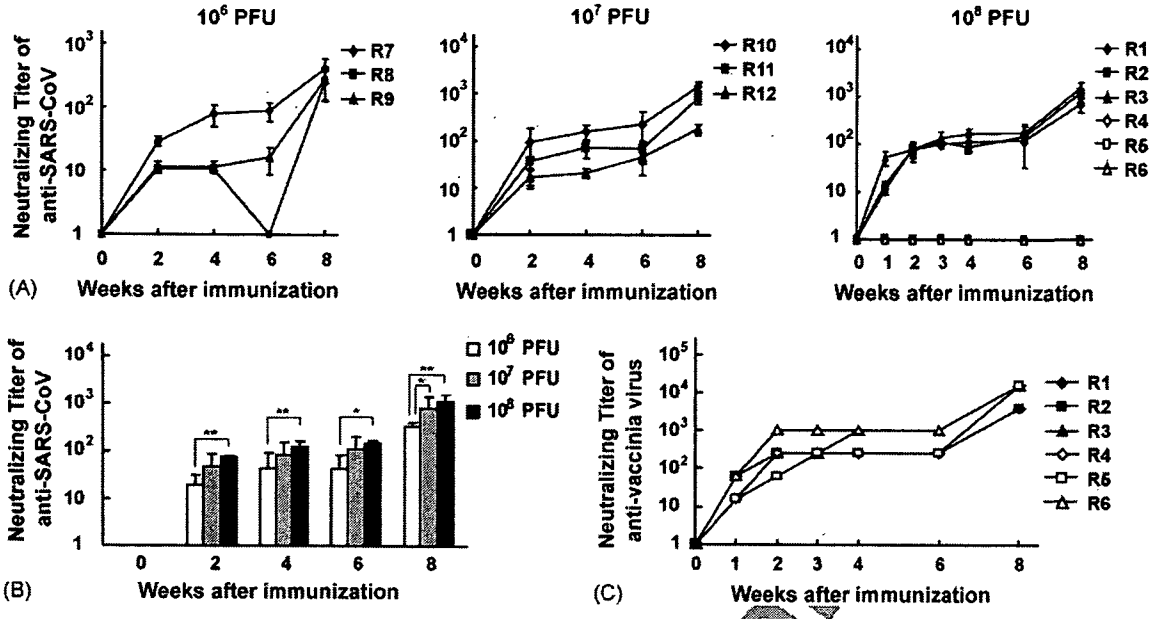

Fig. 3. Induction of NT antibodies against SARS-CoV and vaccinia virus. (A) The NT activity against SARS-CoV of RVV-S- (10^6 PFU, R7–9; 10^7 PFU, R10–12; 10^8 PFU, R1–3; closed symbols) or 10^8 PFU of LC16m8- (R4–6; open symbols) immunized rabbit sera was defined as the maximum dilution of sera that inhibited the cytopathic effect of SARS-CoV by more than 50%. (B) The dose dependency of immunization with RVV-S shown in (A). $^*p<0.05$, $^{**}p<0.01$. (C) The NT activity against vaccinia virus of RVV-S- (R1–3, closed symbols) or LC16m8- (R4–6, open symbols) immunized sera was defined as the maximum dilution of sera that inhibited plaque formation by LC16mO by more than 50%.

4. Discussion

In the present study, we generated a SARS-CoV spike protein-expressing recombinant vaccinia virus using a highly attenuated strain, LC16m8, and demonstrated that NT antibodies against SARS-CoV can be strongly induced by immunization with RVV-S, not only in naïve rabbits but also in LC16m8 pre-immunized rabbits.

In a previous study, passive transfer of sera obtained from mice inoculated with SARS-CoV prevented the replication of SARS-CoV in the upper and lower respiratory tract [29]. In addition, intraperitoneal injection of sera from mice immunized with MVA expressing spike protein (MVA/S) reduced the viral titers in lung and nasal turbinate in a dose-dependent manner [18]. These finding indicate that NT antibodies against spike protein are sufficient to protect against SARS-CoV infection. Single immunization with 10^7 or 10^8 PFU of RVV-S and two immunizations with 10^6 PFU of RVV-S were able to induce a high level of NT antibodies against SARS-CoV at 2 weeks after immunization. Therefore, RVV-S also may protect against SARS-CoV *in vivo* and would be a highly effective vaccine against SARS in naïve individuals.

Contrary to the above studies [18,29], Czub et al. [30] reported that immunization with MVA/S did not prevent SARS-CoV infection in ferrets but rather produced inflammatory responses and focal necrosis in the liver after SARS-CoV challenge. This may have been due to only low NT activity against SARS-CoV being induced by the MVA/S immunization. Moreover, the precise mechanism of this liver inflammation has not been clarified. Feline infectious peritonitis virus (FIPV), another member of the coronaviruses, exhibited enhanced FIPV infection into monocytes/macrophages through viral-specific antibody binding to the Fc receptors of these cells, and caused enhanced inflammation [31]. However, there is no evidence that NT antibodies against SARS-CoV cause antibody-dependent enhancement, and correlation between inflammation and antibody-dependent enhancement by MVA/S vaccination has not yet been established. The side effects of vaccines are also influenced by the dosage and route of immunization. In Czub's report, MVA/S was intraperitoneally injected into the ferrets, although most vaccinations with RVV are conducted through other routes, such as intradermal, intramuscular or subcutaneous injection. Therefore, selection of a different immunization route may prevent such side effects. Nonetheless, further analysis of the side effects of various SARS vaccines, including RVV-S, is required in *in vivo* SARS-CoV challenge models in a variety of animals.

Using RVV-S as a candidate SARS vaccine means that possible complications due to previous vaccination with the VV for smallpox may be avoided. Hammarlund et al. [32] reported that a particular antiviral antibody against poxvirus is maintained for a very long time (possibly for life) by immunization with the smallpox vaccine. Therefore, there was concern that a RVV vaccine would be eliminated by the host antiviral immune response before induction of effective humoral and/or cellular immunity against the protein encoded by the transduced gene. However, immunization with 10^7 PFU of RVV-S induced NT antibodies against SARS-CoV in rabbits that had been immunized with 10^7 PFU

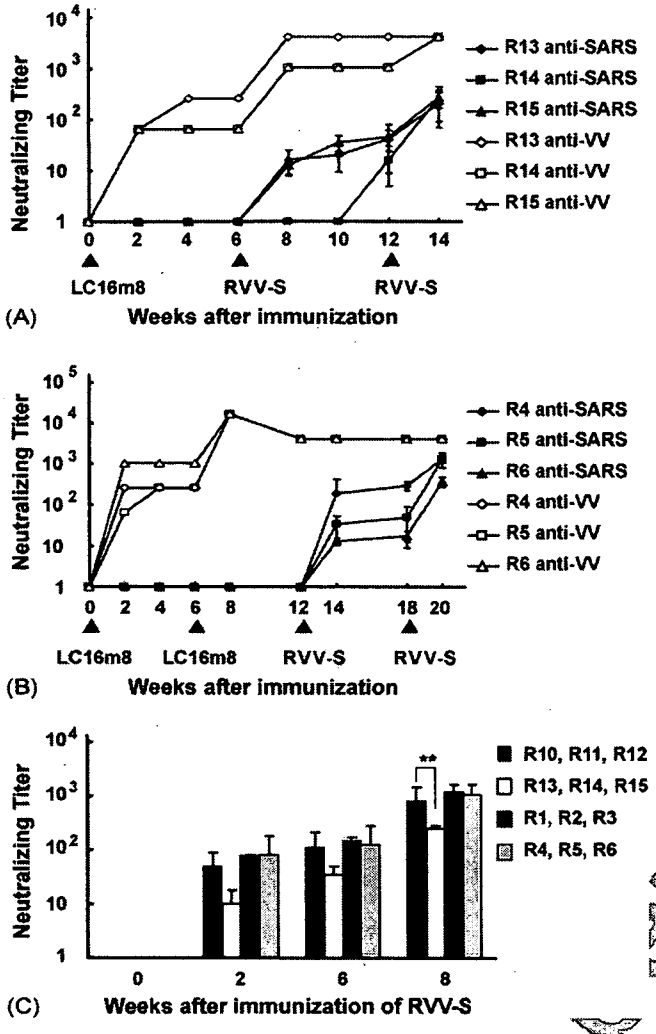

Fig. 4. Induction of NT antibodies against SARS-CoV in rabbits pre-immunized with LC16m8. (A) Three rabbits (R13–15) were immunized with 10^7 PFU of LC16m8 on day 0, and then immunized with 10^7 PFU of RVV-S at 6 and 12 weeks. (B) Three rabbits (R4–6) were immunized with 10^8 PFU of LC16m8 at 0 and 6 weeks, and then immunized with 10^8 PFU of RVV-S at 12 and 18 weeks. Immunized rabbit sera were analyzed by *in vitro* NT assay against SARS-CoV (closed symbols) or vaccinia virus (open symbols). Each type of symbol indicates one and the same individual, and the schedule of immunization with RVV-S or LC16m8 is indicated by arrowheads. (C) Comparison of NT antibodies against SARS-CoV induced by RVV-S in VV-immunized or naïve rabbits. RVV-S (10^7 or 10^8 PFU) was injected into rabbits at 0 and 6 weeks in the presence (10^7 PFU, R13–15; 10^8 PFU, R4–6) or absence (10^7 PFU, R10–12; 10^8 PFU, R1–3) of pre-immunization with an equal titer of LC16m8. Immunized rabbit sera were analyzed by *in vitro* NT assay against SARS-CoV. **$p < 0.01$.

of LC16m8. Since the NT titer against VV induced by 10^7 PFU of LC16m8 was comparable to that in people vaccinated with the smallpox vaccine [32], RVV-S may induce NT antibodies against SARS-CoV in such people. On the other hand, 10^8 PFU of RVV-S also induced NT antibodies against SARS-CoV in rabbits that had an extremely high titer of NT antibodies against VV due to two pre-immunizations with 10^8 PFU of LC16m8. Furthermore, there was no difference in the NT titer against SARS-CoV induced by RVV-S between naïve rabbits and LC16m8 pre-immunized rabbits. The immune response against a protein encoded by a transduced gene may be influenced by the amount of antigen expression, the antigenicity of the protein encoded by a transduced gene, the route of immunization and viral proliferation in the host, and thus further analysis is required to resolve the precise mechanism involved. Furthermore, the vaccine effect of RVV-S still needs to be confirmed in humans pre-immunized with the smallpox vaccine.

In the present study, immunization with RVV-S manifested a vaccine effect against SARS-CoV, in spite of the pre-existing NT antibodies against VV. This finding indicates that an RVV vaccine derived from LC16m8 can be used for people previously immunized with the smallpox vaccine. Furthermore, this RVV vaccine could be repeatedly used against various microbes, such as influenza virus, by alteration of the protein encoded by the transduced gene. Therefore, the use of an RVV vaccine generated from LC16m8 is a promising vaccine strategy against various infectious diseases.

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