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Analysis of the mechanism by which BALB/c mice having prior immunization with nucleocapsid protein of SARS-CoV develop severe pneumonia after SARS-CoV infection

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Abstract

The precise mechanism of severe acute respiratory syndrome (SARS), which is caused by SARS-associated coronavirus (SARS-CoV), is still unclear. We generated recombinant vaccinia virus (rVV) LC16m8 strain which simultaneously expresses four structural proteins of SARS-CoV, including nucleocapsid (N), membrane (M), envelop (E), spike (S) proteins (rVV-NMES) and reported that old BALB/c mice having prior immunization with rVV-NMES develop severe pneumonia similar to those of control mice though rVV-NMES-immunized mice showed lower pulmonary viral titer than in the control mice. Furthermore, we determined which SARS-CoV structural protein for the prior rVV-immunization is responsible for the severe pneumonia after the SARS-CoV infection as observed in the rVV-NMES-immunized mice. Old BALB/c mice were inoculated intradermally with rVV that expressed each structural proteins of SARS-CoV (rVV-N, -M, -E, or -S) with or without rVV-S and then infected intranasally with SARS-CoV more than 4 weeks later. At 9 days after SARS-CoV infection, the rVV-N-immunized mice show more severe pneumonia than in other groups. Furthermore, significant up-regulation of Th1 (IL-2)- and Th2 (IL-4 and IL-5)-bias cytokines and down-regulation of anti-inflammatory cytokine (IL-10 and TGF-β) were observed in rVV-N-immunized mice, resulting in the intensive infiltration of immunocompetent cells into the lung. In contrast, rVV-S-immunized mice showed only low pulmonary viral tier and slight pneumonia. However, the mice having co-immunization with both rVV-N and rVV-S showed severe pneumonia though their pulmonary viral titer was low. These results suggest that an excessive host immune response against the N protein of SARS-CoV is involved in severe pneumonia caused by SARS-CoV infection. These findings increase our understanding of the pathogenesis of SARS.

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1. Introduction

From November 2002 to July 2003, an outbreak of severe acute respiratory syndrome (SARS), which originated in China, spread worldwide, resulting in 8098 cases with 774 deaths (http://www.who.int/csr/sars/country/en/index.html). Patients with SARS usually develop high fever followed by severe clinical symptoms, which include acute respiratory distress syndrome with diffuse alveolar damage (DAD), and ultimately die. A novel type of coronavirus, termed SARS-associated coronavirus (SARS-CoV), was identified as the etiologic agent of SARS (1-3). Angiotensin-converting enzyme 2 (ACE2) was identified as the host receptor for SARS-CoV (4). ACE2 is abundantly expressed in the epithelia of the lung and small intestine and may mediate SARS-CoV entry in humans (5). Although intensive investigations rapidly unraveled the sequence of the SARS-CoV genome and its receptor in humans, the precise molecular mechanism underlying the development of SARS is not fully understood.

The possible roles of host anti-SARS-CoV immune responses have been suggested in severe clinical cases. The uncontrolled release of immune mediators, called a ‘cytokine storm’, has been implicated in the pathogenesis of SARS. However, the cytokine profiles of SARS patient sera do not correlate with the severity of pneumonia because of their diversity. These results might be related to patient anamnesis. Therefore, the development of animal models for SARS is needed to understand the pathogenesis of SARS. Recently, Roberts et al. reported that aged BALB/c mice (older than 12 months) exhibited high and prolonged levels of viral replication, signs of clinical symptoms, and histopathologic changes in the lung (6). Aged BALB/c mice represent a conventional

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animal model that mimics the findings in elderly SARS patients, many of whom exhibit severe disease, requiring intensive care and ventilation support, as well as increased mortality.

In the present study, we investigated the pulmonary immune responses and pathologies of intranasally SARS-CoV-infected BALB/c mice of more than 6 months of age that were previously immunized with SARS-CoV structural proteins using vaccinia virus vectors.

2. Generation of recombinant vaccinia virus

We generated plasmid vector (pBMSF) which expresses transgenes under the control of ATI/p7.5 synthetic hybrid promoter for homologous recombination into the hemagglutinin (HA) locus of vaccinia virus LC16m8 strain (m8) (7). Briefly, the transgenes that encode the SARS-CoV structural proteins were ligated by inserting internal ribosomal entry site (IRES) sequence of hepatitis C virus (2a and 1b/2b) fused with the 2A sequence of Foot and Mouth Disease Virus (FMDV) and *Thosea asigna* virus (TaV) or encephalomyocarditis virus (EMCV) by PCR (Fig. 1A). The generated DNA fragment was digested with *Eco*RI and inserted downstream of the ATI/p7.5 hybrid promoter of pBMSF, thereby generating pBMSF-SARS-NMES. The pBMSF-SARS-NMES plasmid was linearized with *Pvu*I, and transfected into primary rabbit kidney (PRK) cells that had been infected with m8 at a multiplicity of infection (moi) of 10. After 36 h, the virus-cell mixture were harvested by scraping, and frozen at -80ºC until use. The resulting HA-negative recombinant viruses were purified as described previously (8), and named m8rVV-NMES. Furthermore, recombinant vaccinia virus LC16mO (mO) that expressed the SARS-CoV N, M or E protein with a six-histidine tag at the C-terminus was generated (mOrVV-NHis, -MHis, and EHis), as was mO that expressed six-histidine-tagged S protein (mOrVV-SHis), as described previously (8).

3. SARS-CoV challenge experiment

Female BALB/c mice aged more than the 6 months were used in this study. Four groups of BALB/c mice (n=7-8 per group) were inoculated intradermally with either 1×10^7 pfu/body of m8, m8rVV-S or m8rVV-NMES or 70 μl of vehicle (MEM without FCS). At 7-8 wks post-immunization, the mice were infected intranasally with 1×10^5 $TCID_{50}$/body of SARS-CoV (20 μl/mouse), as described previously (9). The mice were sacrificed under anesthesia and the lung, liver, small intestine, and spleen were extirpated 2 days and 9 days later. Aliquots of these tissues were frozen immediately at -80ºC or fixed with 10% formalin. The collected blood was used for the *in vitro* neutralization assay (8). In addition, BALB/c mice were injected intradermally with 1×10^7 pfu/body of rVV that expressed each structural protein of SARS-CoV (mOrVV-N, -M, -E, -S) with or without LC16mOrVV-S (i.e., LC16mOrVV-N, -M, -E, -S alone or LC16mOrVV-N + S, -M + S, -E + S), and infected with 1×10^5 $TCID_{50}$/body of SARS-CoV more than 4 wks later. After 2 days and 9 days, mice (n=3-5 per group) were sacrificed following blood collection under anesthesia, and their lungs were extirpated. All animal experiments using mice were approved by the Animal Experiment Committee at The Institute of Medical Science, University of Tokyo, and were performed in accordance with the animal experimentation guidelines of The Institute of Medical Science, University of Tokyo. The SARS-CoV titers in the mouse organs were determined as described previously (9). Serial 10-fold dilutions of the supernatants of the lung homogenates were added to Vero E6 cells seeded on 96-well plates. After 6 days of incubation, the cells were fixed with 10% formalin. Viral titer was determined as the 50% endpoint dilution of the homogenate that induced the cytopathic effect. The method used for endpoint calculation was that described by Reed and Muench (10). Ten % formalin-fixed lung tissues of the SARS-CoV-infected mice were embedded in paraffin. Paraffin block sections (4-μm thickness) were stained with hematoxylin and eosin (HE staining). We evaluated pulmonary pathology using the histopathologic scoring systems developed by Cimolai et al. (11). To measure the levels of cytokine/chemokine mRNA, total RNA samples were extracted from the lungs using the RNeasy Mini kit (Qiagen). Quantitative RT-PCR was carried out with TaqMan Gene Expression assays (Applied Biosystems, Foster City, CA) using the ABI Prism 7700 and Sequence Detection System software ver. 1.7. The fold-change in copy number of each cytokine/chemokine mRNA was revealed using the $2^{-\Delta\Delta Ct}$ method using 18s rRNA as an endogenous calibrator.

4. Results and Discussion

Using attenuated vaccinia virus strain LC16 m8 (m8), we generated recombinant vaccinia virus having a multicistronic transgene that expresses simultaneously four structural proteins of SARS-CoV (N, M, E, and S proteins). The m8rVV-NMES and m8rVV-S were inoculated intradermally on the backs of BALB/c mice at 1×10^7 pfu/body. At 7-8 wks after this single immunization, the mice were infected intranasally with SARS-CoV at 1×10^5 $TCID_{50}$/body (Fig. 1B). At 9 days after SARS-CoV infection, severe pulmonary inflammation was observed in m8rVV-NMES-immunized BALB/c mice (Fig. 1C, D). However, the initial virus titer was significantly lower than those of the control groups, which included vehicle- and m8-immunized mice (Fig. 1E). The severity of pulmonary inflammation did not correlate with the virus titer in the m8rVV-NMES-immunized mice, in contrast to the correlations observed for the vehicle-, m8-, and m8rVV-S-immunized groups. At 9 days post-infection, both the m8rVV-NMES- and m8rVV-S-immunized groups could elicit significantly neutralizing antibodies against SARS-CoV than in control groups (data not shown) and alleviate SARS-CoV infection. These results suggest that the severe pulmonary inflammation seen in m8rVV-NMES-immunized mice after SARS-CoV infection results from host immune responses rather than a direct cytopathic effect of SARS-CoV, since the virus titers for all the group were

negligible 9 days after SARS-CoV infection and the virus titer of the m8rVV-NMES-immunized group was significantly decreased 2 days post-infection.

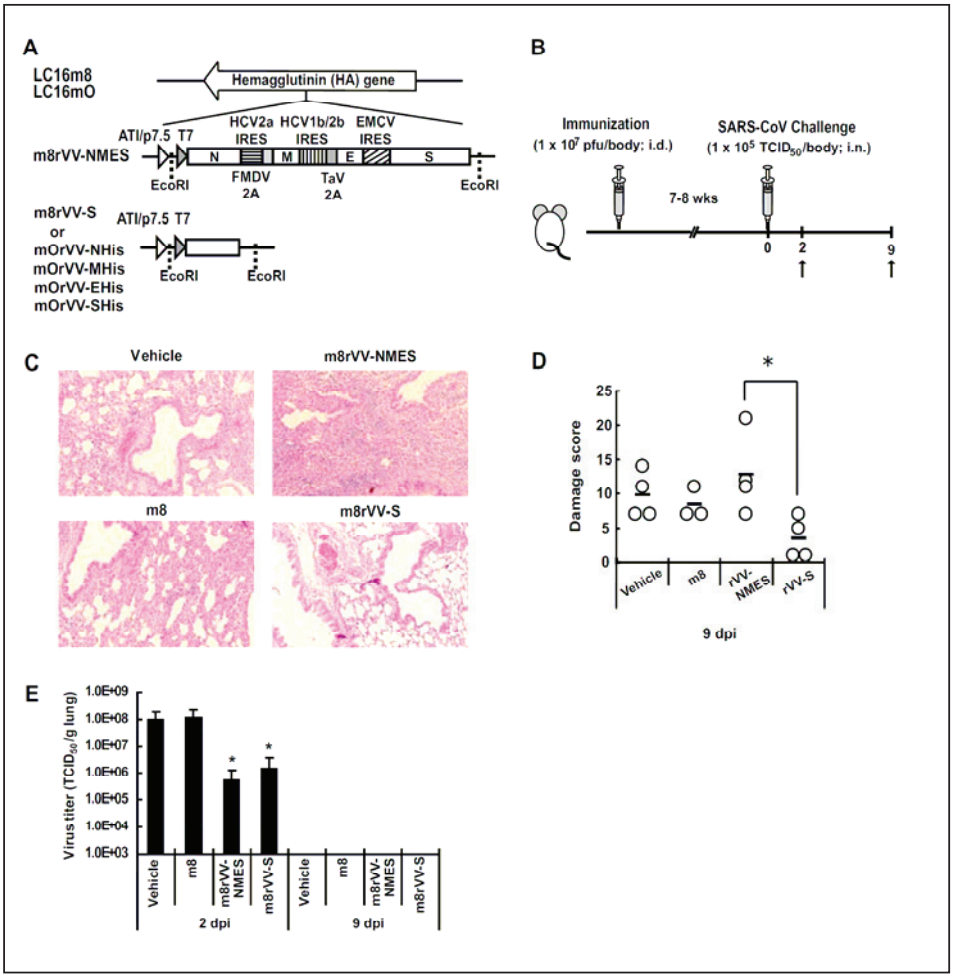

Figure 1. SARS-CoV challenge to BALB/c mice immunized with m8rVV-NMES or m8rVV-S.

(A) Construction of recombinant vaccinia virus that expresses four structural proteins of SARS-CoV (m8rVV-NMES). DNA fragments that encode the SARS-CoV N, M, E, and S proteins were ligated with the IRES sequence as described in *Materials and methods*. Recombinant mO that expressed the SARS-CoV N, M, E or S protein was also generated (mOrVV-NHis, -MHis, -EHis, and -SHis). (B) Four groups of BALB/c mice (n=7-8) were inoculated intradermally with m8rVV-NMES, m8rVV-S, m8 or vehicle, and challenged 7-8 wks later with 1×10^5 $TCID_{50}$/body of SARS-CoV delivered *via* the intranasal route. Blood and lung tissues samples were collected at the indicated time-points. (C) Extirpated lung tissues were fixed with 10% formalin and embedded in paraffin. Paraffin block sections (4-μm thickness) were stained with hematoxylin and eosin (HE staining). (D) The degree of pulmonary inflammation was determined in a blinded fashion on a subjective 27-point scale (0, minimal inflammation; 26, massive inflammation) as described in *Materials and methods*. Each point represents an individual mouse. An asterisk indicates a significant difference. (E) After 2 days and 9 days, the titers of SARS-CoV in the lungs of mice in each group (n=3-4) were determined. Virus titers are expressed as $\log_{10}$ $TCID_{50}$ per gram of tissue.

We hypothesized that the severe pulmonary inflammation seen in the m8rVV-NMES-immunized mice resulted from the host immune responses to SARS-CoV components expressed by m8rVV-NMES. Therefore, we investigated the influence of rVV expressing each structural protein of SARS-CoV (mOrVV-N, -M, -E, and -S) on subsequent intranasal infection with SARS-CoV. BALB/c mice were immunized with mOrVV-N, -M, -E, and -S at 1×10^7 pfu/body, and 4 wks later infected intradermally with 1×10^5 $TCID_{50}$ of SARS-CoV (Fig. 2A). Consistent with the above results, a

significant reduction of pulmonary virus titer was observed after 2 days in only the mOrVV-S-immunized group (Fig. 2B). In contrast, immunization with the other SARS-CoV structural proteins, including the N, M, and E proteins, did not confer protection against the subsequent SARS-CoV infection. The alleviation of pulmonary inflammation was also observed in the mOrVV-S-immunized group (Fig. 2C). Severe infiltration of lymphocytes and thickening of the alveolar epithelia were observed in the lung tissues of the mOrVV-N-immunized mice 9 days after SARS-CoV infection (Fig. 2C). The pulmonary damage in the mOrVV-N-immunized mice was significantly more severe than that in the mOrVV-S-immunized mice (Fig. 2D). However, there were no significant differences among the other groups.

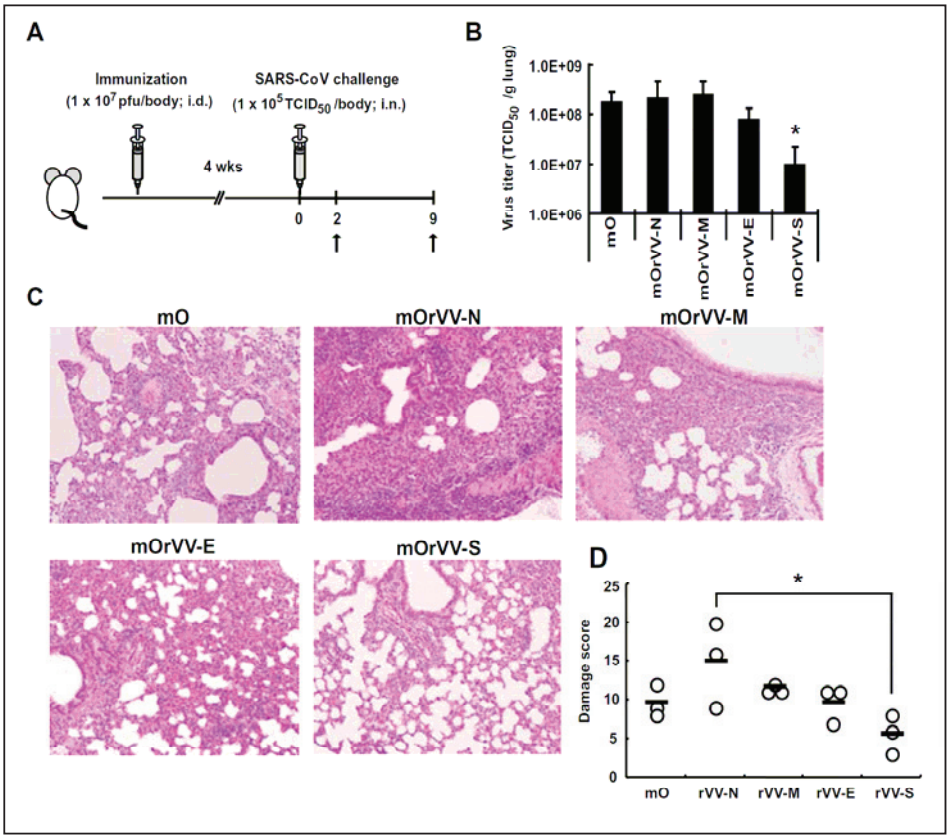

Figure 2. Identification of SARS-CoV structural protein implicated in severe pulmonary inflammation.

(A) Five groups of BALB/c mice were inoculated intradermally with mOrVV-N, -M, -E, -S, or mO, and challenged 4 wks later with 1×10^5 $TCID_{50}$/body of SARS-CoV *via* the intranasal route. (B) After 2 days, the titers of SARS-CoV in the lungs of three mice in each group were determined. *$p<0.05$, as compared to the mO-immunized group using the Dunnett test. (C) Histopathologic findings for all the groups 9 days after SARS-CoV infection. Paraffin block sections (4-μm thickness) were subjected to HE staining. (D) The degree of pulmonary inflammation was determined in a blinded fashion on a subjective 27-point scale (0, minimal inflammation; 26, massive inflammation). Each point represents an individual mouse. An asterisk indicates a significant difference.

To confirm the exacerbating effect of N protein immunization, we investigated the pulmonary virus titers and histopathology in BALB/c mice that were previously immunized with the combination of mOrVV-N and mOrVV-S (mOrVV-N+S-immunized group) 2 days and 9 days after SARS-CoV infection, and compared them to those of all other groups, including the mO-, mOrVV-M+S- mOrVV-E+S-, and mOrVV-S-immunized groups. The mOrVV-N+S-immunized group showed significantly decreased pulmonary virus titers compared to the mO-immunized group (Fig. 3A). However, the mOrVV-N+S-immunized group exhibited as severe pneumonia as the mO-immunized group (Fig. 3B, 3C). In contrast, both the mOrVV-M+S-immunized group and the mOrVV-E+S-immunized group were protected against SARS-CoV infection to the same extent as the mOrVV-S-immunized group (Fig. 3A-C).

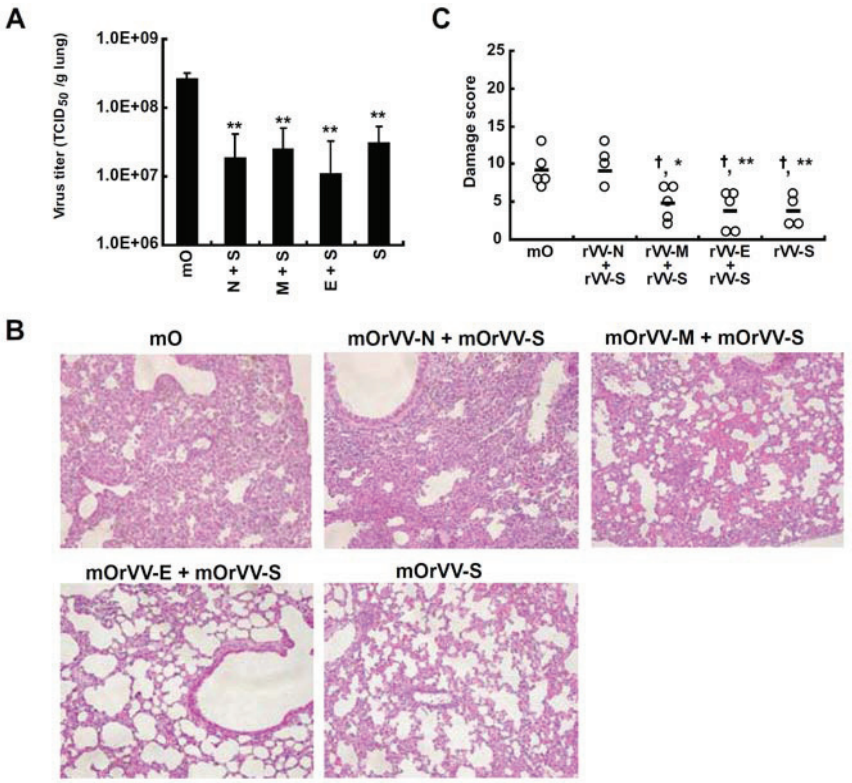

Figure 3. Severe pneumonia in BALB/c mice that were previously immunized with the combination of N protein and S protein of SARS-CoV.

(A) Five groups of BALB/c mice were inoculated intradermally with the combinations of mOrVV-N and mOrVV-S (mOrVV-N+S), mOrVV-M and mOrVV-S (mOrVV-M+S), mOrVV-E and mOrVV-S (mOrVV-E+S), mOrVV-S, and mO, and challenged 7 wks later with 1×10^5 $TCID_{50}$/body of SARS-CoV *via* the intranasal route. After 2 days, the titers of SARS-CoV in the lungs of three to five mice from each group were determined. *p<0.05, **p<0.01 using the Bonferroni test, as compared to the mO-immunized group. (B) Histopathologic findings for all the groups 9 days after SARS-CoV infection. Paraffin block sections (4-μm thickness) were subjected to HE staining. (C) The degree of pulmonary inflammation was determined in a blinded fashion on a subjective 27-point scale (0, minimal inflammation; 26, massive inflammation). Each point represents an individual mouse. †p<0.05, ††p<0.01, as compared to the mO-immunized group using the Bonferroni test. *p<0.05, **p<0.01, as compared to the mOrVV-N + S-immunized group using the Bonferroni test.

To date, no studies have been was reported regarding SARS patients with severe pneumonia who were previously immunized with either SARS-CoV or a highly related species. In contrast, there are several reports of antisera against human coronaviruses (229E and OC43) and host factor IL-11 cross-reacting with the SARS-CoV antigen (12, 13). Furthermore, the N protein of SARS-CoV has been shown to induce both cellular and humoral immune responses (14-16). Taken together, these results raise the possibility that a percentage of SARS patients already possess the adaptive immune response elements that can interact with SARS-CoV components, including the N protein, and that their adaptive immune response may be involved in the exacerbation of pneumonia. The temporal changes in immune response and the pathogenesis after SARS-CoV infection of an animal model that had previously been immunized with SARS-CoV components are not well-understood, as almost all the previous studies reported only protection within a few days of SARS-CoV infection (17, 18). In the present study, we demonstrate that mOrVV-N-immunized mice after SARS-CoV infection exhibit an imbalance between T-cell activation (high expression levels of IFN-γ, IL-2, IL-4, and IL-5) and subsequent suppression (low expression levels of IL-10 and TGF-β), as well as high-level production of proinflammatory cytokines (IL-6 and TNF-α) and chemokines (CCL2, CCL3, and CXCL10) (data not shown). Both IL-10 and TGF-β play important roles in suppressing inflammatory responses (19). Thus, the reduced production of both anti-inflammatory cytokines in the mOrVV-N-immunized mice after SARS-CoV infection may be related to the severity of the pulmonary inflammation in these mice. Feline infectious peritonitis virus (FIPV), which is another member of the coronavirus family, exhibits enhanced FIPV infection into monocytes/macrophages through virus-specific antibody binding to the Fc receptors of these cells, and causes enhanced inflammation (20). It has also been reported for dengue virus that secondary infection with a different genotype results in more severe symptoms, including dengue hemorrhagic fever (DHF) and dengue shock syndrome

(DSS). The exacerbation of this symptom is also positively associated with pre-existing antibodies with specificity for dengue virus (21). In the case of SARS-CoV, antibody-dependent enhancement of infection has not been reported previously. We hypothesized that the severe pneumonia observed in mOrVV-N-immunized mice after SARS-CoV infection does not result from antibody-dependent enhancement, since the virus titers in the mouse lungs 9 days later were below the detection limit. Deming, et al. reported recently the intensive infiltration of eosinophils as well as lymphocytes after SARS-CoV infection of aged BALB/c mice previously immunized with the N protein of SARS-CoV (22). The authors speculated that the Th2-biased responses of vaccinated hosts after SARS-CoV infection might aggravate pulmonary inflammation, although the main host response remains unknown. In contrast, our current data suggest that N protein-immunized mice exhibit activation of both Th1 and Th2 responses after SARS-CoV infection. In agreement with our data, Jin et al. have demonstrated that prior immunization with N protein generates stronger antigen-specific Th1 and Th2 responses than immunization with M or E protein (23). These results suggest that further studies, including epitope analysis, are required to reveal the precise mechanism underlying the severe pulmonary inflammation that results from SARS-CoV infection of BALB/c mice immunized with the N protein of SARS-CoV.

In contrast, intradermal immunization of aged BALB/c mice with m8rVV-S at 1×10^7 pfu/body significantly reduced the pulmonary virus titer 2 days after SARS-CoV infection (Fig. 1E). Furthermore, the m8rVV-S-immunized group exhibited alleviation of the pulmonary histopathology, as compared to both control groups after 9 days (Fig. 1C, D). These results suggest that the systemic immune responses induced by a single immunization with SARS vaccine successfully protect the animal model against intranasal SARS-CoV infection.

In conclusion, we demonstrate that the immunization of BALB/c mice with the N protein of SARS-CoV causes severe pulmonary inflammation upon subsequent SARS-CoV infection, probably *via* the imbalance created between T-cell activation and suppression, as well as by massive proinflammatory cytokine production. These results provide new insights into the mechanisms involved in the pathogenesis of SARS and help in the development of safe vaccines.

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