2. Akdis M, Verhagen J, Taylor A, Karamloo F, Karagiannidis C, Crameri R, Thunberg S, Deniz G, Valenta R, Fiebig H, Kegel C, Disch R, Schmidt-Weber CB, Blaser K, Akdis CA (2004) Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. J Exp Med 199:1567–1575
3. Asseman C, Mauze S, Leach MW, Coffman RL, Powrie F (1999) An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. J Exp Med 190:995–1004
4. Belisle JT, Vissa VD, Sievert T, Takayama K, Brennan PJ, Besra GS (1997) Role of the major antigen of *Mycobacterium tuberculosis* in cell wall biogenesis. Science 276:1420–1422
5. Burney PG, Chinn S, Rona RJ (1990) Has the prevalence of asthma increased in children? Evidence from the national study of health and growth 1973–86. BMJ 300:1306–1310
6. Cavani A, Nasorri F, Ottaviani C, Sebastiani S, De Pita O, Girolomoni G (2003) Human CD25+ regulatory T cells maintain immune tolerance to nickel in healthy, nonallergic individuals. J Immunol 171:5760–5768
7. Cookson WO, Moffatt MF (1997) Asthma: an epidemic in the absence of infection? Science 275:41–42
8. Del Prete G (1992) Human Th1 and Th2 lymphocytes: their role in the pathophysiology of atopy. Allergy 47:450–455
9. Erb KJ, Holloway JW, Sobeck A, Moll H, Le Gros G (1998) Infection of mice with *Mycobacterium bovis*-Bacillus Calmette-Guerin (BCG) suppresses allergen-induced airway eosinophilia. J Exp Med 187:561–569
10. Fontenot JD, Gavin MA, Rudensky AY (2003) Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol 4:330–336
11. Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, de Vries JE, Roncarolo MG (1997) A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature 389:737–742
12. Herz U, Gerhold K, Gruber C, Braun A, Wahn U, Renz H, Paul K (1998) BCG infection suppresses allergic sensitization and development of increased airway reactivity in an animal model. J Allergy Clin Immunol 102:867–874
13. Horwitz DA, Zheng SG, Gray JD (2003) The role of the combination of IL-2 and TGF-beta or IL-10 in the generation and function of CD4+ CD25+ and CD8+ regulatory T cell subsets. J Leukoc Biol 74:471–478
14. Hunt JR, Martinelli R, Adams VC, Rook GA, Brunet LR (2005) Intragastric administration of *Mycobacterium vaccae* inhibits severe pulmonary allergic inflammation in a mouse model. Clin Exp Allergy 35:685–690
15. Inoue J, Aramaki Y (2007) Suppression of skin lesions by transdermal application of CpG-oligodeoxynucleotides in NC/Nga mice, a model of human atopic dermatitis. J Immunol 178:584–591
16. Inoue Y, Isobe M, Shiohara T, Goto Y, Hayashi H (2002) Protective and curative effects of topically applied CX-659S, a novel diaminouracil derivative, on chronic picryl chloride-induced contact hypersensitivity responses. Br J Dermatol 147:675–682
17. Kariyone A, Higuchi K, Yamamoto S, Nagasaka-Kametaka A, Harada M, Takahashi A, Harada N, Ogasawara K, Takatsu K (1999) Identification of amino acid residues of the T-cell epitope of *Mycobacterium tuberculosis* alpha antigen critical for Vbeta11(+) Th1 cells. Infect Immun 67:4312–4319
18. Kariyone A, Tamura T, Kano H, Iwakura Y, Takeda K, Akira S, Takatsu K (2003) Immunogenicity of Peptide-25 of Ag85B in Th1 development: role of IFN-gamma. Int Immunol 15:1183–1194
19. Kitagaki H, Fujisawa S, Watanabe K, Hayakawa K, Shiohara T (1995) Immediate-type hypersensitivity response followed by a late reaction is induced by repeated epicutaneous application of contact sensitizing agents in mice. J Invest Dermatol 105:749–755
20. Kitagaki H, Ono N, Hayakawa K, Kitazawa T, Watanabe K, Shiohara T (1997) Repeated elicitation of contact hypersensitivity induces a shift in cutaneous cytokine milieu from a T helper cell type 1 to a T helper cell type 2 profile. J Immunol 159:2484–2491
21. Kitagaki H, Kimishima M, Teraki Y, Hayakawa J, Hayakawa K, Fujisawa S, Shiohara T (1999) Distinct in vivo and in vitro cytokine profiles of draining lymph node cells in acute and chronic phases of contact hypersensitivity: importance of a type 2 cytokine-rich cutaneous milieu for the development of an early-type response in the chronic phase. J Immunol 163:1265–1273
22. Kuromatsu I, Matsuo K, Takamura S, Kim G, Takebe Y, Kawamura J, Yasutomi Y (2001) Induction of effective antitumor immune responses in a mouse bladder tumor model by using DNA of an alpha antigen from mycobacteria. Cancer Gene Ther 8:483–490
23. Ling EM, Smith T, Nguyen XD, Pridgeon C, Dallman M, Arbery J, Carr VA, Robinson DS (2004) Relation of CD4+CD25+ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. Lancet 363:608–615
24. McKnight AJ, Zimmer GJ, Fogelman I, Wolf SF, Abbas AK (1994) Effects of IL-12 on helper T cell-dependent immune responses in vivo. J Immunol 152:2172–2179
25. Overbergh L, Valckx D, Waer M, Mathieu C (1999) Quantification of murine cytokine mRNAs using real time quantitative reverse transcriptase PCR. Cytokine 11:305–312
26. Powrie F, Carlino J, Leach MW, Mauze S, Coffman RL (1996) A critical role for transforming growth factor-beta but not interleukin 4 in the suppression of T helper type 1-mediated colitis by CD45RB(low) CD4+ T cells. J Exp Med 183:2669–2674
27. Sakaguchi S, Sakaguchi N, Shimizu J, Yamazaki S, Sakihama T, Itoh M, Kuniyasu Y, Nomura T, Toda M, Takahashi T (2001) Immunologic tolerance maintained by CD25+ CD4+ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. Immunol Rev 182:18–32
28. Shirakawa T, Enomoto T, Shimazu S, Hopkin JM (1997) The inverse association between tuberculin responses and atopic disorder. Science 275:77–79
29. Swain SL, Weinberg AD, English M, Huston G (1990) IL-4 directs the development of Th2-like helper effectors. J Immunol 145:3796–3806
30. Takamura S, Matsuo K, Takebe Y, Yasutomi Y (2005) Ag85B of mycobacteria elicits effective CTL responses through activation of robust Th1 immunity as a novel adjuvant in DNA vaccine. J Immunol 175:2541–2547
31. Takatsu K, Kariyone A (2003) The immunogenic peptide for Th1 development. Int Immunopharmacol 3:783–800
32. Tamura T, Matsubara M, Hasegawa K, Ohmori K, Karasawa A (2005) Olopatadine hydrochloride suppresses the rebound phenomenon after discontinuation of treatment with a topical steroid in mice with chronic contact hypersensitivity. Clin Exp Allergy 35:97–103
33. Teixeira FM, Teixeira HC, Ferreira AP, Rodrigues MF, Azevedo V, Macedo GC, Oliveira SC (2006) DNA vaccine using *Mycobacterium bovis* Ag85B antigen induces partial protection against experimental infection in BALB/c mice. Clin Vaccine Immunol 13:930–935
34. Ulmer JB, Montgomery DL, Tang A, Zhu L, Deck RR, DeWitt C, Denis O, Orme I, Content J, Huygen K (1998) DNA vaccines against tuberculosis. Novartis Found Symp 217:239–246 discussion 246–253
35. Yazdanbakhsh M, Kremsner PG, van Ree R (2002) Allergy, parasites, and the hygiene hypothesis. Science 296:490–494
36. Zhu D, Jiang S, Luo X (2005) Therapeutic effects of Ag85B and MPT64 DNA vaccines in a murine model of *Mycobacterium tuberculosis* infection. Vaccine 23:4619–4624
37. Zuany-Amorim C, Sawicka E, Manlius C, Le Moine A, Brunet LR, Kemeny DM, Bowen G, Rook G, Walker C (2002) Suppression of airway eosinophilia by killed *Mycobacterium vaccae*-induced allergen-specific regulatory T-cells. Nat Med 8:625–629

Prior Immunization with Severe Acute Respiratory Syndrome (SARS)-Associated Coronavirus (SARS-CoV) Nucleocapsid Protein Causes Severe Pneumonia in Mice Infected with SARS-CoV[1]

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The details of the mechanism by which severe acute respiratory syndrome-associated coronavirus (SARS-CoV) causes severe pneumonia are unclear. We investigated the immune responses and pathologies of SARS-CoV-infected BALB/c mice that were immunized intradermally with recombinant vaccinia virus (VV) that expressed either the SARS-CoV spike (S) protein (LC16m8rVV-S) or simultaneously all the structural proteins, including the nucleocapsid (N), membrane (M), envelope (E), and S proteins (LC16m8rVV-NMES) 7–8 wk before intranasal SARS-CoV infection. The LC16m8rVV-NMES-immunized group exhibited as severe pneumonia as the control groups, although LC16m8rVV-NMES significantly decreased the pulmonary SARS-CoV titer to the same extent as LC16m8rVV-S. To identify the cause of the exacerbated pneumonia, BALB/c mice were immunized with recombinant VV that expressed the individual structural proteins of SARS-CoV (LC16mOrVV-N, -M, -E, -S) with or without LC16mOrVV-S (i.e., LC16mOrVV-N, LC16mOrVV-M, LC16mOrVV-E, or LC16mOrVV-S alone or LC16mOrVV-N + LC16mOrVV-S, LC16mOrVV-M + LC16mOrVV-S, or LC16mOrVV-E + LC16mOrVV-S), and infected with SARS-CoV more than 4 wk later. Both LC16mOrVV-N-immunized mice and LC16mOrVV-N + LC16mOrVV-S-immunized mice exhibited severe pneumonia. Furthermore, LC16mOrVV-N-immunized mice upon infection exhibited significant up-regulation of both Th1 (IFN-γ, IL-2) and Th2 (IL-4, IL-5) cytokines and down-regulation of anti-inflammatory cytokines (IL-10, TGF-β), resulting in robust infiltration of neutrophils, eosinophils, and lymphocytes into the lung, as well as thickening of the alveolar epithelium. These results suggest that an excessive host immune response against the nucleocapsid protein of SARS-CoV is involved in severe pneumonia caused by SARS-CoV infection. These findings increase our understanding of the pathogenesis of SARS. *The Journal of Immunology,* **2008, 181: 6337–6348.**

From November 2002 to July 2003, an outbreak of severe acute respiratory syndrome (SARS),[4] 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, and ultimately death. A novel type of coronavirus (CoV), termed SARS-associated CoV (SARS-CoV), was identified as the etiologic agent of SARS (1–3). The genome of SARS-CoV is a single strand of positive-sense RNA of ~30 kb in length with 14 putative open reading frames, which encode nonstructural replicase polyproteins and several structural proteins, including spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins (4). The S protein of SARS-CoV, like the S proteins of other CoVs, plays an important role in the first step of viral infection by binding to a host cell receptor. Angiotensin-converting enzyme 2 was identified as the host receptor for SARS-CoV (5). Angiotensin-converting enzyme 2 is abundantly expressed in the epithelia of the lung and small intestine and may mediate SARS-CoV entry in humans (6). 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

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[4] Abbreviations used in this paper: SARS, severe acute respiratory syndrome; CoV, coronavirus; VV, vaccinia virus; HA, hemagglutinin; MOI, multiplicity of infection; VLP, virus-like particle; $TCID_{50}$, tissue culture ID_{50}.

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. For example, Jones et al. (7) have reported a decreased number of IL-2-, IL-4-, IL-10-, and IL-12-producing cells in SARS-CoV-infected patients. In contrast, Wong et al. (8) have demonstrated increased production of IFN-γ, IL-1, IL-6, and IL-12 p70, but not of IL-2, IL-4, IL-10 or TNF-α, which is consistent with a Th1 response. The data from these adult patients with SARS show no clear trend toward either a Th1 or Th2 bias. These results might be related to patient anamnesis. Therefore, the development of animal models for SARS is needed to understand the pathogenesis of SARS. Non-human primates, mice, ferrets, and hamsters have been found to support the replication of SARS-CoV (9–14). However, an animal model that mimics the clinical symptoms and pathology observed in SARS patients has not been reported to date. Recently, Roberts et al. (15) reported that aged BALB/c mice (older than 12 mo) exhibited high and prolonged levels of viral replication, signs of clinical symptoms, and histopathologic changes in the lung. Aged BALB/c mice represent a conventional 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 older than 6 mo of age that were previously immunized with SARS-CoV structural proteins using vaccinia virus (VV) vectors, by measuring various cytokine mRNAs and histopathologies of the lungs.

Materials and Methods

Cells and viruses

RK13 cells (CCL-37) from the American Type Culture Collection (ATCC) and Vero E6 cells (CRL-1586) from ATCC were cultured in MEM (Nissui Pharmaceutical) that contained 5% FCS. To generate recombinant VV LC16m8, which expresses the structural proteins of SARS-CoV, primary rabbit kidney cell cultures were prepared by overnight digestion with 100 PU/ml dispase (Sanko Jun-yaku) of kidneys extirpated from 7-day-old inbred JW rabbits (Kitayama Labs). The cells were grown in T175 flasks in lactalbumin medium with Hank's salts (LH) that contained 5% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. When the cell confluency was ~50%, the culture medium was replaced with lactalbumin medium with Eagle's salts (LE) that contained 5% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. SARS-CoV Vietnam/NB-04/2003 strain, which was isolated from the throat wash fluid of one patient (16), was provided by Dr. M. Quynh Le. VVs LC16m8 (m8) and LC16mO (mO) were provided by the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan). All work using SARS-CoV was performed in BioSafety Level 3 facilities by personnel wearing powered air-purifying respirators (Shigematsu Works).

Generation of recombinant VV

To generate a pBR322-based plasmid vector (pBMSF) for homologous recombination into the hemagglutinin (HA) locus of m8, we cloned the HA gene, which contained the ATI/p7.5 synthetic hybrid promoter, from the pSFJ1–10 plasmid and inserted it into the pBM vector, which was reconstructed in our laboratory. Full-length cDNAs for the SARS-CoV nucleocapsid (N), membrane (M), and envelope (E) proteins were cloned from the Vietnam/NB-04/2003 strain of SARS-CoV by RT-PCR (16). Full-length SARS-CoV spike (S) protein gene was prepared from pSFJ1-10-SARS-S, which is described in our previous report (17). Next, the genes that encode the SARS-CoV structural proteins were ligated by inserting internal ribosomal entry site sequence of hepatitis C virus (genotypes 2a and 1b/2b) fused with the 2A sequence of foot and mouth disease virus and *Thosea asigna* virus or encephalomyocarditis virus by PCR (see Fig. 1A). The generated DNA fragment was digested with *Eco*RI and inserted downstream of the ATI/p7.5 hybrid promoter of pBR322-based plasmid vector pBMSF, thereby generating pBMSF-SARS-NMES. The pBMSF-SARS-NMES plasmid was linearized with *Pvu*I, and transfected into primary rabbit kidney 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 −80°C until use. The resulting HA-negative recombinant viruses were purified as previously described (17), and named m8rVV-NMES. Furthermore, recombinant mO that expressed the SARS-CoV N, M, or E protein with a six histidine tag at the C terminus was generated (mOrVV-NHis, mOrVV-MHis, and mOrVV-EHis), as was mO that expressed six histidine-tagged S protein (mOrVV-SHis), as previously described (17).

Western blot analysis

Vero E6 cells were infected with m8rVV-NMES at an MOI of 5. After 18 h, the cells were lysed with lysis buffer (10 mM Tris (pH 7.4), 150 mM NaCl, 1% SDS, 0.5% Nonidet P-40, protease inhibitor cocktail). The cell lysates (30 μg) were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore). After blocking the membranes with 5% skim milk solution at room temperature for 1 h, the membrane was incubated with polyclonal Abs against the N, M, E, or S protein. Vero E6 cell lysates infected with mOrVV-NHis, mOrVV-MHis, mOrVV-EHis, or mOrVV-SHis was used as positive controls. We used the anti-S polyclonal Abs described in our previous study (17). Polyclonal Abs against N and E proteins were prepared from rabbit sera immunized with KLH-conjugated N peptide (residues aa 250–263) and E peptide (residues aa 61–73). Polyclonal Abs against the M protein were provided by Dr. Mizutani (National Institute of Infectious Diseases, Musashimurayama, Tokyo). We purified the IgG fractions of these antisera using the protein A Ampure PA kit (Amersham Biosciences). After washing with TBS that contained 0.1% Tween 20 (TBST), the membranes were reacted with HRP-conjugated $F(ab')_2$ of anti-rabbit IgG (GE Healthcare). Each specific protein band was visualized using the ECL system (GE Healthcare).

Indirect immunofluorescence analysis

Vero E6 cells were infected with m8rVV-NMES at an MOI of 5 at 30°C for 4 h. The cells were washed with PBS and fixed with cold acetone/methanol (1/1) mixture for 10 min. After blocking with TNB blocking buffer (NEN Life Science Products) at room temperature for 1 h, the fixed cells were incubated with polyclonal Abs against the N, M, or E protein or mAb against the S protein (designated as anti-S-His protein, clone no. 13B8), which was originally prepared in our laboratory, at 4°C overnight. After washing, the cells were incubated with Alexa Fluor 488-conjugated anti-rabbit IgG or mouse IgG Ab at room temperature for 1 h. Nuclei were stained with DAPI (4',6-diamidino-2-phenylindole). Fluorescence images were acquired using a confocal microscope (LSM510 META; Carl Zeiss).

Confirmation of SARS-CoV-like particle formation

RK13 cells were cultured in 150-mm dishes, and then infected with m8rVV-NMES at an MOI of 5. After 48 h of incubation, the culture supernatants were collected and centrifuged to remove cell debris at 3000 rpm for 30 min at 4°C. The supernatants were concentrated ~100-fold using the Pellicon XL (cut off molecular weight 3×10^5; Millipore). The isolation of virus-like particles (VLP) was performed as previously described, with a slight modification (18). Briefly, the concentrated supernatant was placed on 60% (w/w) sucrose cushion and centrifuged at 4.0×10^4 rpm for 5 h. The opalescent band was collected and centrifuged in a 20–60% (w/w) sucrose gradient at 2.7×10^4 rpm for 4 h, and then divided into 20 fractions. The protein content of each fraction was determined with the DC protein assay kit (Bio-Rad). The 20 μl of each fraction were separated by SDS-PAGE (7.5%, 10%, or 15% polyacrylamide gel), and transferred onto a polyvinylidene difluoride membrane. The membrane was incubated with mAb against S protein (13B8), mAb against N protein (IMG-654; Imgenex) or polyclonal Abs against the M or E protein. After washing, the membranes were reacted and visualized as described. The VLPs in the concentrated culture supernatant were visualized using transmission electron microscopy. For immunogold staining, VLPs were loaded onto a collodion-coated electron microscopy grid for 5 min. After the removal of excess sample solution, polyclonal Ab against S protein was added onto the grid and incubated at room temperature for 1 h. The grids were washed six times with Sorensen's phosphate buffer at room temperature and incubated with 5-nm gold-conjugated anti-rabbit IgG for 1 h. After washing with Sorensen's phosphate buffer for 10 s, the samples were stained with 2% phosphotungstic acid for 1 min. After draining off the excess phosphotungstic acid, the samples were observed under the electron microscopy.

Immunization of rabbits with m8rVV-NMES

Groups of three New Zealand White rabbits (SLC) were immunized intradermally with 1×10^8 PFU/body of m8rVV-NMES or with 1×10^8 PFU/body of m8, at 0 and 6 wk. Sera were collected at the indicated time

points (see Fig. 2A), and used in ELISA analysis and in the in vitro neutralization assay described below. All animal experiments using rabbits were approved by The Tokyo Metropolitan Institute of Medical Science Animal Experiment Committee and were performed in accordance with the animal experimentation guidelines of The Tokyo Metropolitan Institute of Medical Science.

ELISA

Recombinant SARS-CoV N, M, E, and S proteins tagged with six histidines at the C terminus were expressed in RK13 cells by infecting with mOrVV-N-His, mOrVV-E-His, mOrVV-M-His, or mOrVV-S-His at an MOI of 5. These proteins were purified using nickel Sepharose (6 Fast Flow; GE Healthcare). His-tagged E and M proteins were further purified by SDS-PAGE. These full-length structural proteins (0.2 μg/ml, 50 μl/well) were coated onto 96-well plates at 4°C overnight. The plates were blocked with 1% BSA in PBS(−) that contained 0.5% Tween 20 and 2.5 mM EDTA, and then incubated with serial 2-fold dilutions of sera from the rabbits immunized with m8rVV-NMES or m8. After extensive washing, the plates were assayed as previously described, except that *o*-phenylenediamine was used as the substrate (17). The individual SARS-CoV structural protein-specific IgG titers are presented as the end point dilution Ab titers. The end point titer was defined as the reciprocal of the highest dilution of serum at which the absorbance at 490 nm (A_{490}) ratio (A_{490} of m8rVV-NMES-immunized serum/A_{490} of m8-immunized serum (negative control)) was greater than 2.0, as previously described (19).

In vitro neutralization assay for SARS-CoV

The neutralizing Ab titers of the sera of rabbits immunized with m8rVV-NMES or m8 were determined as previously described (17). Briefly, serial 2-fold dilutions of heat-inactivated sera were mixed with equal volumes of 200 tissue culture ID_{50} ($TCID_{50}$) of SARS-CoV and incubated at 37°C for 1 h. Vero E6 cells were then infected with 100 μl of the virus-serum mixtures in 96-well plates. After 5 days (or 6 days in the SARS-CoV challenge experiment) of infection, the neutralization titer was determined as the end point dilution of the serum at which there was 50% inhibition of the SARS-CoV-induced cytopathic effect. The method used for end point calculation was that described by Reed and Muench (20).

SARS-CoV challenge experiment

Female BALB/c mice older than the 6 mo of age (SLC) were used in this study. Four groups of eight BALB/c mice (seven mice in the vehicle-immunized 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 wk postimmunization, the mice were infected intranasally with 1×10^5 $TCID_{50}$/body of SARS-CoV (20 μl/mouse), as previously described (11). Four mice from each group were sacrificed 2 and 9 days later, except for the three mice of the vehicle-immunized group, which were sacrificed 2 days later. The mice were sacrificed under anesthesia and the lung, liver, small intestine, and spleen were extirpated. Aliquots of these tissues were frozen immediately at −80°C or fixed with 10% formalin. The collected blood was used for the in vitro neutralization assay. In addition, BALB/c mice were injected intradermally with 1×10^7 PFU/body of recombinant VV that expressed each structural protein of SARS-CoV (mOrVV-NHis, mOrVV-MHis, mOrVV-EHis, mOrVV-SHis) with or without LC16mOrVV-SHis (i.e., LC16mOrVV-N, -M, -E, -S alone or LC16mOrVV-N + LC16mOrVV-S, -M + LC16mOrVV-S, or -E + LC16mOrVV-S), and infected with 1×10^5 $TCID_{50}$/body of SARS-CoV more than 4 wk later. After 2 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.

Determination of viral titers in the organs

The SARS-CoV titers in the mouse organs were determined as previously described (11). Briefly, tissue samples (i.e., lung, liver, small intestine, and spleen) were homogenized in a 10-fold volume of Leibovitz 15 medium (Invitrogen). The homogenates were centrifuged at 2000 rpm for 10 min at 4°C. Serial 10-fold dilutions of the supernatants of these 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% end point dilution of the homogenate that induced the cytopathic effect. The method used for end point calculation was that described by Reed and Muench (20).

Lung histopathology and inflammation scores

In accordance with a previous report (11), 10% formalin-fixed lung tissues of the SARS-CoV-infected mice were embedded in paraffin. Paraffin block sections (4-μm thickness) were stained with H&E staining. The peribronchial and perivascular scores were recorded in a blinded fashion by a pathologist. We evaluated pulmonary pathology using the histopathologic scoring systems developed by Cimolai et al. (21), in which the scoring system is weighted heavily for bronchial lesions. This scoring system allowed us to differentiate the severity of pulmonary pathology in small groups of animals. The pathology grading system consisted of a numerical score ranging from 0 to 26. In brief, each section was scored based upon a cumulative total from five categories that incorporated evaluations of the following: A) number of bronchiolar and bronchial sites affected by the periluminal infiltrate (range, 0 to 3); B) severity of the periluminal infiltrate (range, 0 to 3); C) luminal exudate severity (range, 0 to 2); D) frequency of perivascular infiltrate (range, 0 to 3); and E) severity of parenchymal pneumonia (range, 0 to 5). The accumulated numeric score was derived from the sum of the subscores: A+3(B+C)+D+E. Eosinophils were detected in tissue sections by method of Luna (22).

Extraction of total RNA and quantitative RT-PCR of cytokine or chemokine mRNA

To measure the levels of cytokine or chemokine mRNA, total RNA samples were extracted from the lungs using the RNeasy Mini kit (Qiagen). Quantitative RT-PCR was conducted with TaqMan Gene Expression assays (Applied Biosystems) using the ABI Prism 7700 and Sequence Detection System software v.1.7. The fold change in copy number of each cytokine/chemokine mRNA was revealed using the $2^{-\Delta\Delta C_t}$ method using 18 S rRNA as an endogenous calibrator.

Statistical analysis

Data are presented as mean ± SD. Statistical analysis was performed by one-way ANOVA, followed by the Dunnett or Bonferroni test. A value of $p < 0.05$ was considered to be statistically significant.

Results

Generation of recombinant VV that expresses the structural proteins of SARS-CoV

A multicistronic transgene that expresses simultaneously four structural proteins (N, M, E, and S proteins) of SARS-CoV was constructed and inserted into the HA locus of LC16m8 (m8) by homologous recombination (Fig. 1A). Expression of the transgene was placed under the control of the powerful ATI/p7.5 hybrid promoter. We screened for m8rVV-NMES using the erythrocyte agglutination assay (17), and confirmed the insertion of the transgene by PCR. Expression of the N, M, E, and S proteins in Vero E6 cells infected with m8rVV-NMES was detected by Western blot analysis. Recombinant LC16mO (mO) expressing the C-terminal histidine-tagged N, M, E or S protein (mOrVV-NHis, -MHis, -EHis, and -SHis) was generated as previously described, and used as a positive control for each protein. We also used m8rVV-S (17). As shown in Fig. 1B, the expression levels of the N and S proteins in the m8rVV-NMES-infected cells were high and moderate, respectively. In contrast, the expression levels of the M and E proteins in m8rVV-NMES-infected cells were weaker than those in mOrVV-MHis- and mOrVV-EHis-infected cells. The M protein in the m8rVV-NMES-infected cells was 20 kDa, whereas that in the mOrVV-MHis-infected cells was observed as forms of ~20 kDa (nonglycosylated form) and 25 kDa (glycosylated form) (23). Furthermore, we investigated the cellular localizations of these structural proteins by indirect immunofluorescence (Fig. 1C). In m8rVV-NMES-infected cells, all of the SARS-CoV proteins were localized in the perinuclear regions. In particular, the localization of the N protein in m8rVV-NMES-infected cells was different from that in mOrVV-NHis-infected cells, in which the N-His protein was found diffusely in the cytoplasm. VLPs are formed by the assembly of structural proteins in the cytoplasm, followed by release into the culture medium. By infecting m8rVV-NMES into RK13 cells, we confirmed

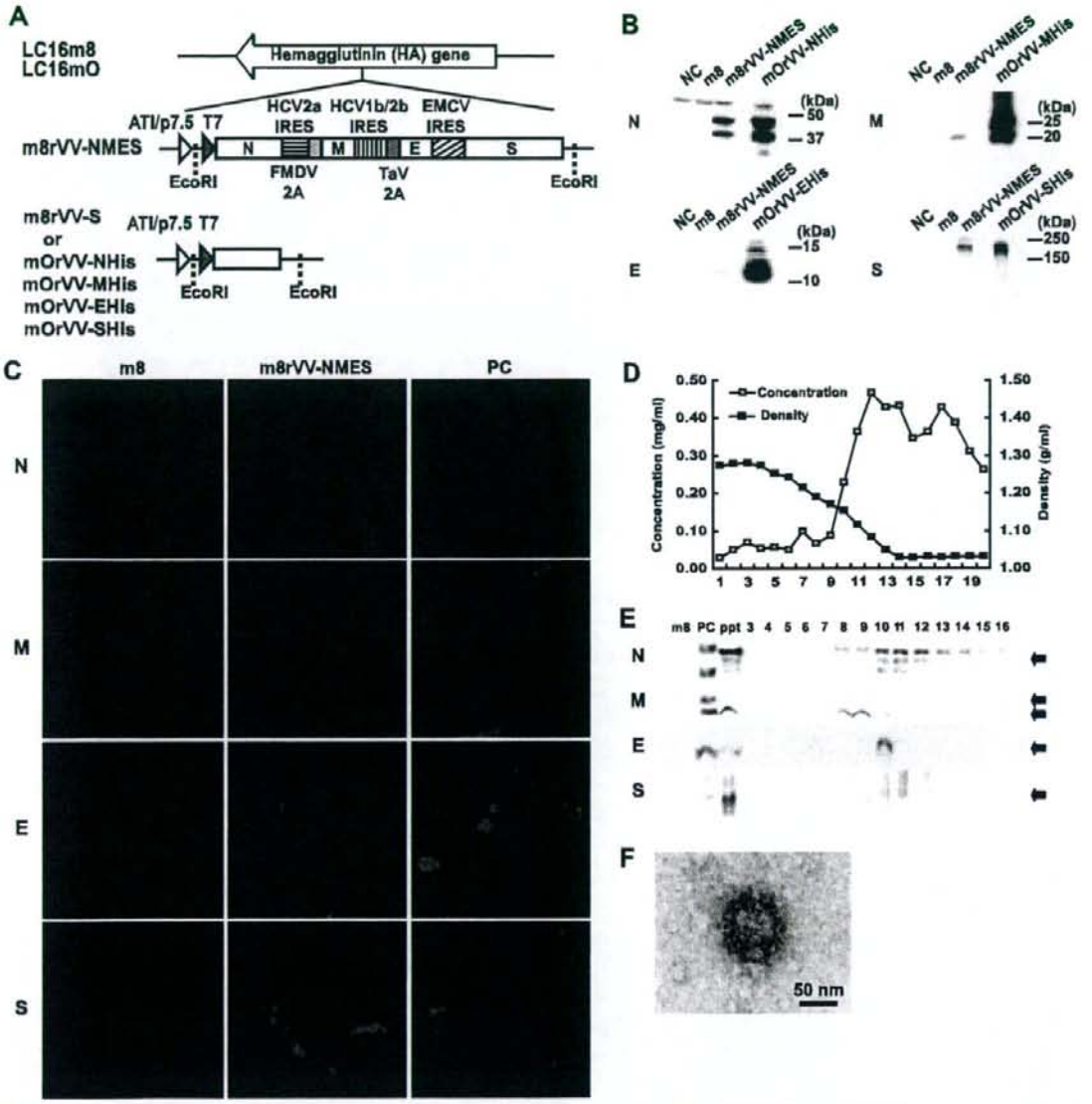

FIGURE 1. Construction of recombinant VV that express four structural proteins of SARS-CoV (m8rVV-NMES). *A*, DNA fragments that encode the SARS-CoV N, M, E, and S proteins were ligated with the internal ribosomal entry site sequence of hepatitis C virus (2a and 1b/2b) and fused with the 2A sequences of foot and mouth disease virus (FMDV) and *Thosea asigna* virus (TaV) or encephalomyocarditis (EMCV). After digestion with *Eco*RI, the DNA fragment was inserted into the pBMSF vector, and the resultant plasmid was designated as pBMSF-NMES. *Pvu*I-linearized pBMSF-NMES was used for homologous recombination into the HA locus of the LC16m8 genome. Recombinant mO that expressed the SARS-CoV N, M, E, or S protein was generated (mOrVV-NHis, -MHis, -EHis, and -SHis) as described in *Materials and Methods*. *B*, Vero E6 cells were infected with m8rVV-NMES or m8. Uninfected Vero E6 cells were used as a negative control (NC). Structural proteins mOrVV-NHis, mOrVV-MHis, mOrVV-EHis, and mOrVV-SHis were used as positive controls. SARS-CoV structural proteins were detected using rabbit polyclonal Abs and donkey anti-rabbit IgG polyclonal Abs, which were conjugated with HRP. The lane between m8rVV-NMES and the mOrVV-N, mOrVV-M, mOrVV-E, and mOrVV-S samples was left empty, to exclude the possibility of leakage of sample solution between lanes. *C*, Vero E6 cells were infected with m8rVV-NMES at an MOI of 5 at 30°C for 4 h. The SARS-CoV proteins in the fixed cells were visualized with the polyclonal Abs against the N, M, or E protein or mAb against the S protein (designated as 13B8). Nuclei were stained with DAPI). Structural proteins mOrVV-NHis, mOrVV-MHis, mOrVV-EHis, and mOrVV-SHis were used as positive controls (PC). *D*, The VLPs were isolated from the culture supernatants of RK13 cells infected with m8rVV-NMES at an MOI of 5 for 48 h at 30°C. After sucrose gradient centrifugation, 20 fractions were collected. *E*, Equal amounts of the gradient fractions (nos. 3–16) were examined by Western blot analyses. m8, m8-infected RK13 cell lysate; ppt, m8rVV-NMES-infected RK13cell lysate; PC, RK13 cell lysates infected with mOrVV-NHis, mOrVV-MHis, mOrVV-EHis, or mOrVV-SHis. *F*, A concentrated culture supernatant was subjected to transmission electron microscopy. VLPs were probed with polyclonal Ab against the S protein and incubated with 5-nm gold-conjugated anti-rabbit IgG.

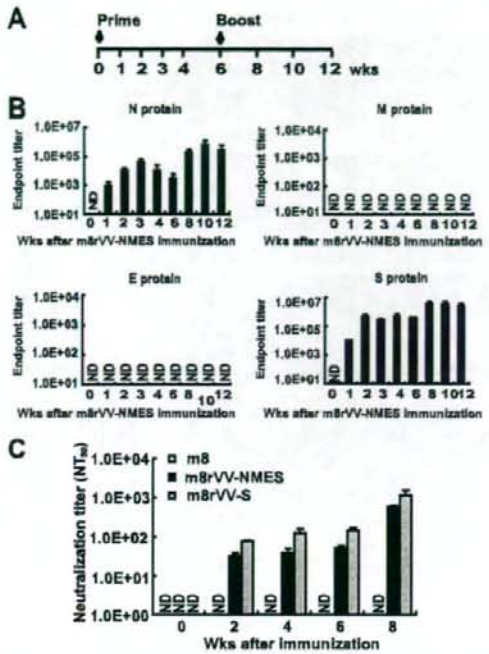

FIGURE 2. Immunogenicity of m8rVV-NMES in rabbits. *A*, New Zealand White rabbits ($n = 3$) were inoculated intradermally with 10^8 PFU/body of m8rVV-NMES or m8 at 0 and 6 wk. Blood samples were collected at the indicated time points. *B*, Induction of serum IgG specific for the four structural proteins of SARS-CoV. The individual SARS-CoV structural protein-specific IgG titers are presented as the end point dilution Ab titers. The end point titer was defined as the reciprocal of the highest dilution of serum at which the absorbance at 490 nm (A_{490}) ratio (A_{490} of m8rVV-NMES-immunized serum/A_{490} of m8-immunized serum (negative control)) was greater than 2.0. *C*, Induction of neutralizing Abs against SARS-CoV. The neutralization titer of m8rVV-NMES-immunized rabbit sera was defined as the end point dilution of the serum at which there was 50% inhibition (NT_{50}) of the SARS-CoV-induced cytopathic effect. Immunization with m8rVVs or m8 was conducted using the schedule described in Fig. 3*A*. ND, Not detectable.

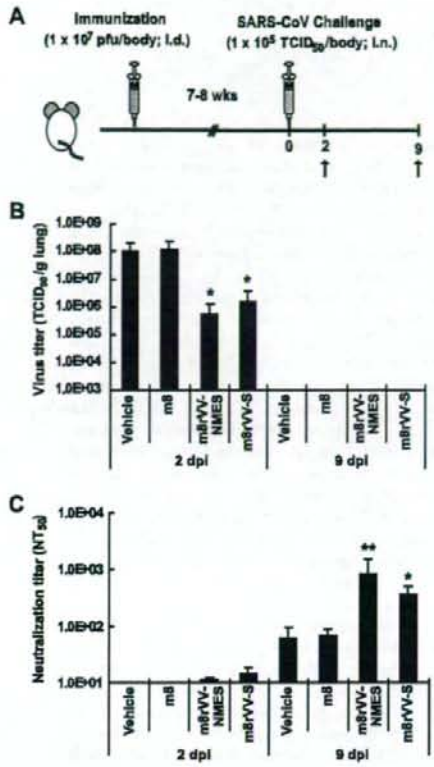

FIGURE 3. SARS-CoV challenge to BALB/c mice immunized with m8rVV-NMES or m8rVV-S. *A*, Four groups of eight BALB/c mice (seven mice in the vehicle-immunized group) were inoculated intradermally with m8rVV-NMES, m8rVV-S, m8, or vehicle and challenged 7–8 wk later with 1×10^5 $TCID_{50}$/body of SARS-CoV delivered via the intranasal route. Blood and lung tissues samples were collected at the indicated time points. *B*, After 2 and 9 days, the titers of SARS-CoV in the lungs of four mice in each group (except for three mice of the vehicle-immunized group, which were examined 2 days later) were determined. Virus titers are expressed as $\log_{10}$ $TCID_{50}$/g of tissue. *C*, At 2 and 9 days after SARS-CoV infection, the serum neutralization titers of all groups were measured as described in *Materials and Methods*. *, $p < 0.05$; **, $p < 0.01$, as compared with both the vehicle- and m8-immunized groups.

the formation of VLPs in the culture medium. After sucrose gradient centrifugation, 20 fractions (500 μl each) were collected (Fig. 1*D*). The four SARS-CoV structural proteins were monitored by Western blot analysis. As shown in Fig. 1*E*, fraction number 10 contained all the SARS-CoV proteins, and the buoyant density of this fraction was ~1.15 g/ml, a value that is consistent with previous reports (18, 24, 25). Moreover, we confirmed the formation of VLPs in the concentrated culture supernatant using scanning electron microscopy and immunogold-labeling with the anti-S protein polyclonal Ab. The particles were 70–100 nm in diameter, which is consistent with the sizes as reported previously (18, 24, 25). The particles were positively stained with immunogold (Fig. 1*F*).

Induction of Abs specific for SARS-CoV structural proteins in rabbits immunized with m8rVV-NMES

To investigate the immunogenicity of m8rVV-NMES, 1×10^8 PFU/body of either m8rVV-NMES or m8, its parental strain, was inoculated intradermally on the backs of New Zealand White rabbits at 0 and 6 wk (Fig. 2*A*). Rabbit antisera specific for the full-length structural proteins of SARS-CoV were detected by ELISA (Fig. 2*B*). In agreement with previous reports (26–28), the N and S proteins both exhibited strong immunogenicity in rabbits. IgG-specific for the N or S protein was induced as early as 1 wk after m8rVV-NMES immunization, and the titer exceeded 1:10000 2 wk later. The titers of Abs against the N and S proteins were dramatically increased by booster immunization with m8rVV-NMES. It was also observed that the Ab titer of the N protein, but not that of the S protein, decreased after reaching the peak titer. Immunization with m8rVV-NMES did not induce Abs specific for the E and M proteins, even after booster immunization (Fig. 2*B*). The antigenicity of the purified E and M proteins coated onto the ELISA plates was confirmed using each rabbit anti-E or anti-M peptide Ab (data not shown). Therefore, we believe that the lack of induction of Abs specific for the E and M proteins in the rabbit sera results from the poor immunogenicity and lower expression levels of these proteins.

Induction of SARS-CoV-neutralizing serum Abs in rabbits by immunizing with m8rVV-NMES

We determined the neutralization titers against SARS-CoV using the same rabbit antisera. The neutralization titer was ~1:30

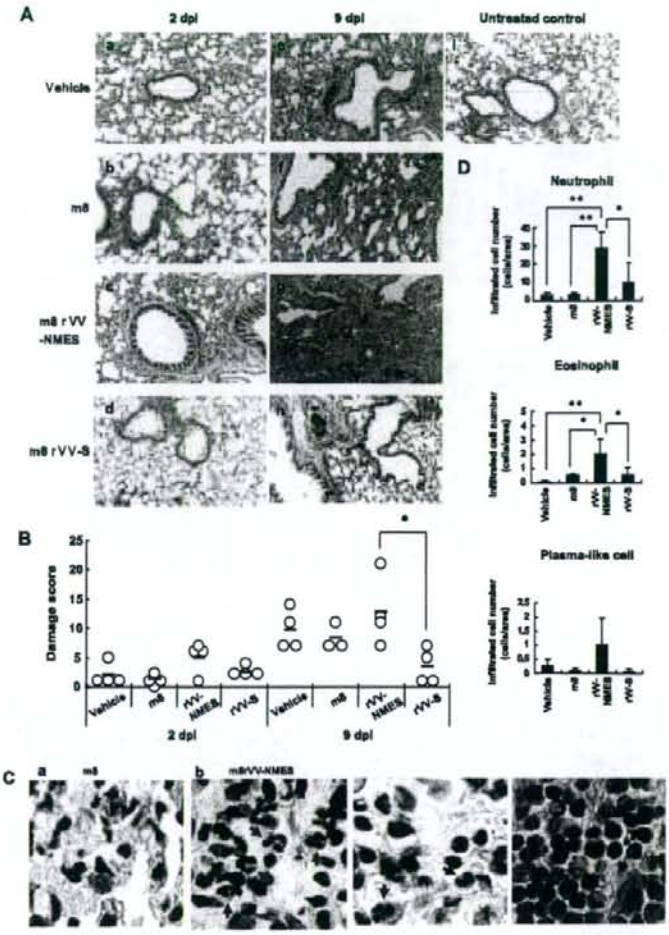

FIGURE 4. Pulmonary histopathology of m8rVV-S-preimmunized BALB/c mice after SARS-CoV challenge. At 7–8 wk after immunization with m8rVV-NMES, m8rVV-S, m8, or vehicle, the mice were infected intranasally with 1×10^5 $TCID_{50}$/body of SARS-CoV. *A*, Four mice from each group (three mice from the vehicle-immunized group were killed 2 days later) were sacrificed 2 and 9 days later. Extirpated lung tissues were fixed with 10% formalin and embedded in paraffin. Paraffin block sections (4-μm thickness) were stained with H&E staining. Histopathologic sections were prepared for vehicle-immunized mice at 2 days postinfection (dpi) (a) and 9 dpi (e), m8-immunized mice at 2 dpi (b) and 9 dpi (f), m8rVV-NMES-immunized mice at 2 dpi (c) and 9 dpi (g), m8rVV-S-immunized mice at 2 dpi (d) and 9 dpi (h), and uninfected mice (i). *B*, 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 symbol represents an individual mouse. *, $p < 0.05$. *C*, Representative lung sections from m8-immunized mice (a) and m8rVV-NMES-immunized mice (b) after staining with Luna method (for eosinophils and neutrophils) and H&E (for plasma cells). Arrows indicate neutrophils (yellow), eosinophils (red), and plasma-like cells (green). *D*, The numbers of neutrophils, eosinophils, and plasma-like cells that infiltrated the lung were counted using Luna method and H&E staining. Data are mean ± SD for $n = 5$ mice. Fields viewed at a magnification of ×400. *, $p < 0.05$; **, $p < 0.01$, for significant differences evaluated using the Bonferroni test.

(range, 1:25 to 1:36) after 2 wk, and was sustained for 6 wk (Fig. 2*C*). Booster immunization with m8rVV-NMES further increased the neutralization titer more than 10-fold 2 wk later. These values are somewhat lower than those induced by m8rVV-S in our previous report (17). In contrast, the antisera from rabbits immunized with m8 did not exhibit any neutralizing activity against SARS-CoV (Fig. 2*C*).

SARS-CoV challenge of BALB/c mice having prior immunization with m8rVV-NMES or m8rVV-S

As m8rVV-NMES and m8rVV-S could induce high levels of neutralizing Abs against SARS-CoV (Fig. 2*C*), we investigated the influences of m8rVV-NMES and m8rVV-S on SARS-CoV challenge of BALB/c mice (Fig. 3*A*). The m8rVV-NMES and m8rVV-S constructs were inoculated intradermally on the backs of BALB/c mice at 1×10^7 PFU/body. At 7–8 wk after this single immunization, the mice were infected intranasally with SARS-CoV at 1×10^5 $TCID_{50}$/body. After 2 and 9 days, the lung, liver, small intestine, and spleen were extirpated from the mice under anesthesia, and the SARS-CoV titers were measured. As shown in Fig. 3*B*, 200- and 100-fold reductions in pulmonary virus titers were observed in the m8rVV-NMES-immunized and m8rVV-S-immunized groups 2 days after infection. The virus titers in the lungs of the m8rVV-NMES-immunized and m8rVV-S-immunized groups were 5.40×10^5 and 1.52×10^6 $TCID_{50}$/g of lung, respectively. In contrast, the vehicle-immunized and LC16m8-immunized groups exhibited virus titers of 1.07×10^8 and 1.18×10^8 $TCID_{50}$/g of lung, respectively. The virus was not detected in the lungs of any group 9 days later, as reported previously (11, 15). In contrast, virus titers in other organs, including liver, small intestine, and spleen, were lower than that of the detection limit 2 and 9 days after infection (data not shown).

We also measured the neutralization titers in these mice sera 2 and 9 days after SARS-CoV infection (Fig. 3*C*). Two days postinfection, the neutralization titers of the m8rVV-NMES-immunized and m8rVV-S-immunized groups were 1:11.1 ± 1.01 and 1:14 ± 3.94, respectively, whereas those of the negative control groups were below the limit of detection. At 9 days postinfection, the serum neutralization titers of m8rVV-NMES-immunized and m8rVV-S-immunized groups had increased to 1:838.0 ± 681.0 and 1:367.9 ± 132.1, respectively. In contrast, the serum neutralizing titers of the vehicle-immunized and m8-immunized groups were 1:59.7 ± 35.4 and 1:67.8 ± 18.6, respectively. These results suggest that both the m8rVV-NMES- and m8rVV-S-immunized groups could elicit neutralizing Abs against SARS-CoV and alleviate SARS-CoV infection.

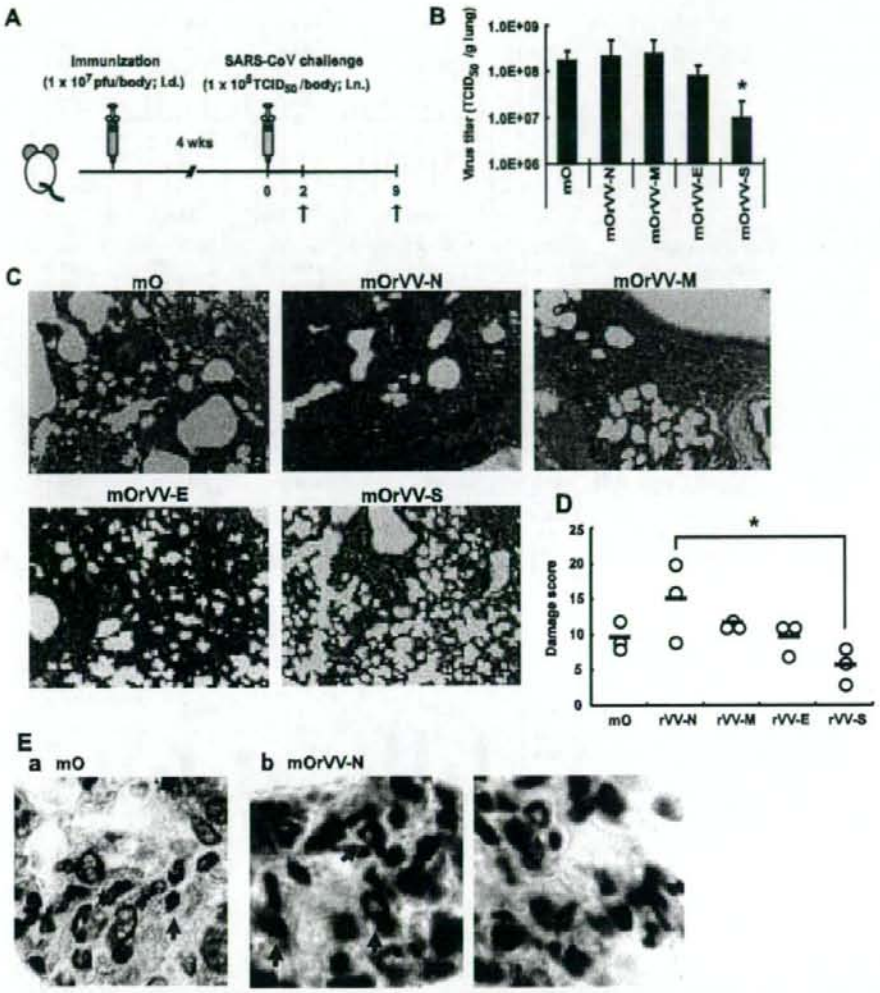

FIGURE 5. Identification of SARS-CoV structural protein implicated in severe pulmonary inflammation. *A*, Five groups of six BALB/c mice were inoculated intradermally with mOrVV-NHis, mOrVV-MHis, mOrVV-EHis, mOrVV-SHis, or mO and challenged 4 wk later with 1×10^5 $TCID_{50}$/body of SARS-CoV via the intranasal route. *B*, After 2 days, the titers of SARS-CoV in the lungs of three mice in each group were determined. Virus titers are expressed as $\log_{10}$ $TCID_{50}$/g of tissue. *, $p < 0.05$, as compared with the mO-immunized group using the Dunnett test. *C*, Histopathologic findings for all the groups 9 days after SARS-CoV infection. Extirpated lung tissues were fixed with 10% formalin and embedded in paraffin. Paraffin block sections (4-μm thickness) were subjected to H&E 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 symbol represents an individual mouse. *, $p < 0.05$. *E*, Representative lung sections from mO-immunized mice (a) and mOrVV-N-immunized mice (b) after staining with Luna method (for eosinophils and neutrophils). Arrows indicate neutrophils (yellow) and eosinophils (red).

Histopathologic findings in the lungs of m8rVVs-immunized BALB/c mice after SARS-CoV infection

We performed histopathologic analyses of lung tissues. Two days after SARS-CoV infection, the vehicle-, m8-, and m8rVV-S-immunized groups showed only slight pulmonary inflammation (Fig. 4*A*, a, b, and d), whereas the m8rVV-NMES-immunized group showed infiltration of lymphocytes into the areas surrounding the bronchi and slight thickening of the alveolar epithelium (Fig. 4*A*, c). We scored pulmonary inflammation in all the groups 2 days after SARS-CoV infection as follows (Fig. 4*B*): in the m8rVV-NMES-immunized group, 5.00 ± 2.71; in the vehicle-immunized group, 2.00 ± 2.00; in the m8-immunized group, 1.33 ± 0.82; and in the m8rVV-S-immunized group, 2.50 ± 1.00. At 9 days postinfection, the vehicle-, m8-, and m8rVV-NMES-immunized groups exhibited severe pulmonary inflammation, i.e., infiltration of inflammatory cells and thickening of alveolar epithelia (Fig. 4*A*, e, f, and g). In contrast, the m8rVV-S-immunized group showed only slight pulmonary inflammation (Fig. 4*A*, h). As shown in Fig. 4*B*, the pulmonary inflammation score for the m8rVV-NMES-immunized group (12.75 ± 2.87) 9 days after SARS-CoV infection was significantly higher than that for the m8rVV-S-immunized group (3.50 ± 3.00). In contrast, this score was comparable to those obtained for the vehicle-immunized and m8-immunized groups (9.75 ± 2.87 and

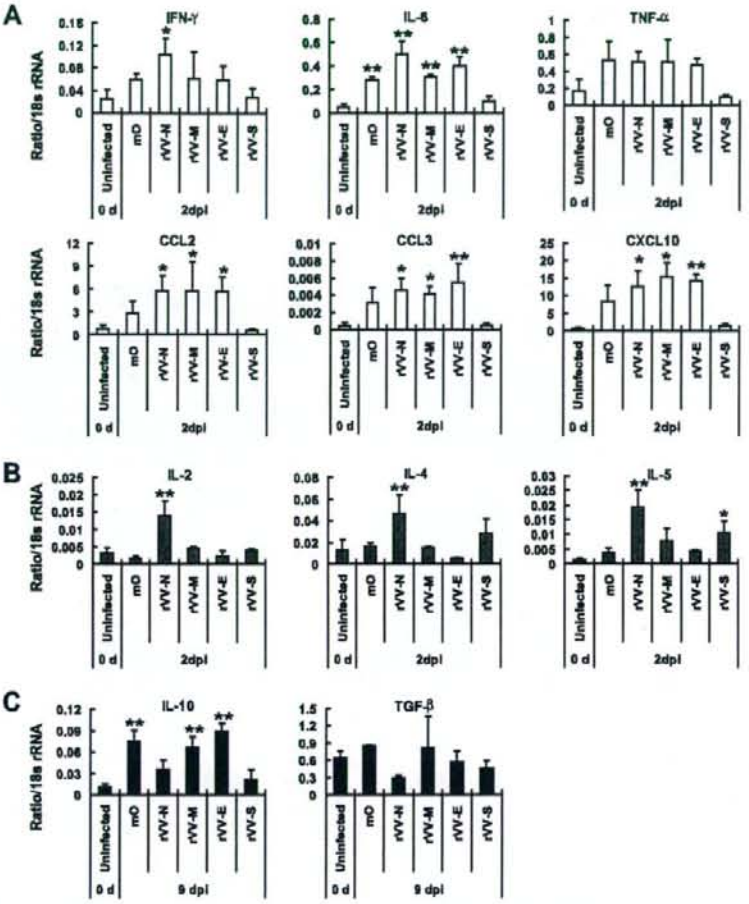

FIGURE 6. Cytokine profiles of the lungs of BALB/c mice preimmunized with each SARS-CoV structural protein and challenged with SARS-CoV. Three mice from each group were sacrificed 2 and 9 days postinfection. The total RNA of the lung was extracted. Quantitative RT-PCR was conducted as described in *Materials and Methods*. The fold change in copy number of each cytokine or chemokine mRNA was calculated by the $2^{-\Delta\Delta C_T}$ method using 18 S rRNA as an endogenous calibrator. *, $p < 0.05$; **, $p < 0.01$, as compared with the uninfected control group using the Bonferroni test. *A*, The levels of mRNA for proinflammatory cytokines and chemokines 2 days after SARS-CoV infection. *B*, The mRNA expression levels of cytokines related to T cell activation 2 days after SARS-CoV infection. *C*, The mRNA expression levels of anti-inflammatory cytokines 9 days after SARS-CoV infection.

8.33 ± 2.31, respectively). The m8rVV-NMES-immunized group exhibited as severe inflammation as the control groups, although m8rVV-NMES contains the S protein and protects as well as m8rVV-S against SARS-CoV infection. In addition, marked infiltration of neutrophils, eosinophils, plasma-like cells, and lymphocytes was observed in the m8rVV-NMES-immunized group, as compared with the control groups, after SARS-CoV infection (Fig. 4*C*, b and *D*).

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, because 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 postinfection.

Identification of the factor that results in the exacerbation of pulmonary inflammation in m8rVV-NMES-immunized BALB/c mice after SARS-CoV infection

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. This notion was supported by the observation of negligible virus titers 9 days after SARS-CoV infection. Therefore, we investigated the influence of recombinant VV expressing each structural protein of SARS-CoV (mOrVV-NHis, mOrVV-MHis, mOrVV-EHis, and mOrVV-SHis) on subsequent intranasal infection with SARS-CoV. BALB/c mice were immunized with mOrVV-NHis, -MHis, -EHis, and -SHis at 1×10^7 PFU/body, and 4 wk later infected intradermally with 1×10^5 $TCID_{50}$ of SARS-CoV (Fig. 5*A*). After 2 and 9 days, three mice from each group were sacrificed following blood collection under anesthesia, and their lungs were extirpated. Consistent with earlier results, a significant reduction of pulmonary virus titer was observed after 2 days in only the mOrVV-SHis-immunized group (Fig. 5*B*). 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. As shown in Fig. 5*C*, the alleviation of pulmonary inflammation was also observed in the mOrVV-SHis-immunized group. Severe infiltration of lymphocytes and thickening of the alveolar epithelia were observed in the lung tissues of the mOrVV-NHis-immunized mice 9 days after SARS-CoV infection (Fig. 5*C*). The pulmonary damage in the mOrVV-NHis-immunized mice (15.00 ± 5.56) was significantly more severe than that in the mOrVV-SHis-immunized mice (5.67 ± 2.52) (Fig. 5*D*). However, there were no significant differences among the other groups. Furthermore, infiltration of neutrophils, eosinophils, and lymphocytes was observed in the

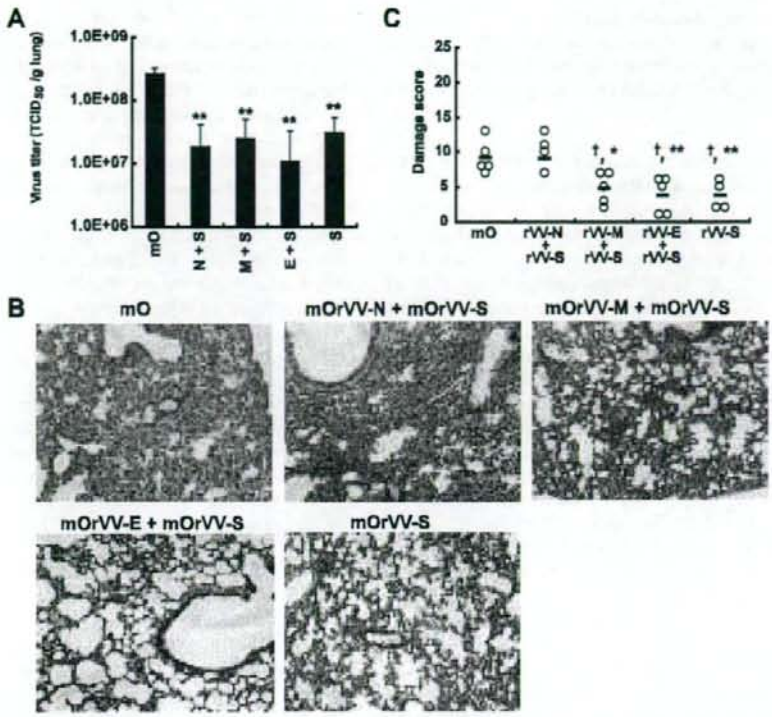

FIGURE 7. Severe pneumonia in BALB/c mice that were previously immunized with the combination of N protein and S protein of SARS-CoV. *A*, Five groups of BALB/c mice (n = 8–10 per group) were inoculated intradermally with the combinations of mOrVV-NHis and mOrVV-SHis (mOrVV-N+S), mOrVV-MHis and mOrVV-SHis (mOrVV-M+S), mOrVV-EHis and mOrVV-SHis (mOrVV-E+S), mOrVV-SHis, and mO, and challenged 7 wk 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 n = 3–5 mice from each group were determined. Virus titers are expressed as $\log_{10}$ $TCID_{50}$/g of tissue. *, $p < 0.05$, **, $p < 0.01$, as compared with the mO-immunized group using the Bonferroni test. *B*, Histopathologic findings for all the groups 9 days after SARS-CoV infection. Extirpated lung tissues were fixed with 10% formalin and embedded in paraffin. Paraffin block sections (4-μm thickness) were subjected to H&E 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 symbol represents an individual mouse. †, $p < 0.05$; ‡, $p < 0.01$, as compared with the mO-immunized group using the Bonferroni test. *, $p < 0.05$; **, $p < 0.01$, as compared with the mOrVV-N + S-immunized group using the Bonferroni test.

mOrVV-NHis-immunized mice after SARS-CoV infection (Fig. 5*E*, b), although the extent of infiltration of these cells into the lungs of these mice was somewhat lower than that observed in the m8rVV-NMES-immunized mice after SARS-CoV infection (Fig. 4*D*). This may explain the observed differences in the histopathologic findings for the mOrVV-NHis-immunized mice and m8rVV-NMES-immunized mice.

Pulmonary cytokine responses of SARS-CoV-infected BALB/c mice previously immunized with recombinant VV expressing each structural protein of SARS-CoV

To elucidate the reason for the severe pulmonary inflammation observed in the mOrVV-NHis-immunized mice after SARS-CoV infection, we measured by quantitative RT-PCR the mRNA levels for various cytokines and chemokines in the lungs of BALB/c mice preimmunized with mOrVV-NHis, -MHis, -EHis, -SHis, or mO. Several proinflammatory cytokine and chemokine mRNAs, including those for IL-6, CXCL10, CCL2, and CCL3, were increased in all the groups, with the exception of the mOrVV-SHis group, 2 days after SARS-CoV infection (Fig. 6*A*). In contrast, the mOrVV-SHis-immunized group showed low levels of mRNA expression for these proinflammatory cytokines or chemokines, especially IL-6, resulting in reduced lung pathology after immunization. The mRNA levels for IFN-γ, IL-2, IL-4, and IL-5 were highest in the mOrVV-NHis-immunized group (Fig. 6, *A* and *B*). None of the other groups showed up-regulation of these cytokines, with the exception of the IL-5 mRNA level in the mOrVV-SHis-immunized group. Furthermore, the mRNA expression levels of anti-inflammatory cytokines (IL-10 and TGF-β) in the mOrVV-NHis-immunized group were markedly lower than expression levels in any of the other groups, which exhibited high virus titers, and were comparable to those of the mOrVV-SHis group, in which pulmonary inflammation was alleviated (Fig. 6*C*).

Verification of exacerbating effect of prior immunization with N protein in SARS-CoV-infected Balb/c mice

To verify 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 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 with the mO-immunized group (Fig. 7*A*). However, the mOrVV-N+S-immunized group exhibited as

severe pneumonia as the mO-immunized group (Fig. 7, *B* and *C*). 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. 7, *A–C*).

Discussion

SARS-CoV is newly identified as an agent of SARS. However, the detailed mechanism by which SARS-CoV causes severe pneumonia remains unclear. The uncontrolled release of immune mediators has been implicated in the pathogenesis of SARS, whereas the cytokine profiles of SARS patients have not elucidated the cause the pneumonia owing to their diversity. It seems likely that the diverse cytokine profiles noted among adult SARS patients are related to patient anamnesis.

In the present study, we observed severe pulmonary inflammation in m8rVV-NMES-immunized BALB/c mice 9 days after SARS-CoV infection (Fig. 4*A*, g), even though the initial virus titer was significantly lower than those of the control groups, which included vehicle- and m8-immunized mice (Fig. 3*B*). 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. We identified the N protein of SARS-CoV as the cause of the severe pneumonia observed during SARS-CoV infection (Fig. 5, *C* and *D*, and 7, *B* and *C*). To date, no studies have been reported to our knowledge 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 CoV (229E and OC43) and host factor IL-11 cross-reacting with the SARS-CoV Ag (29, 30). Furthermore, the N protein of SARS-CoV has been shown to induce both cellular and humoral immune responses (31–33). 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 (34–39). In the present study, we demonstrate that mOrVV-NHis-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). Jiang et al. (40) reported elevation of CXCL10 or IP-10 production in the pneumocytes, $CD3^+$ T cells, and monocytes and macrophages of the lungs of patients with SARS. CXCL10 may be responsible for the infiltration of activated T cells and monocytes or macrophages, which is a pathologic finding in SARS patients (41–43). It has been reported that elevated expression of monocyte or macrophage activation factors (CCL2 and CCL3) was observed in SARS patients (8, 44). Furthermore, the highest expression of IL-6 in mOrVV-NHis-immunized mice is reasonable (Fig. 6*A*), as the elevation of IL-6 levels is considered one of the causes in the severe pneumonia of SARS patients. Zhang et al. (45) reported recently the molecular mechanism of IL-6 expression induction by the N protein of SARS-CoV. In contrast, both IL-10 and TGF-β play important roles in suppressing inflammatory responses (46). Thus, the reduced production of both anti-inflammatory cytokines in the mOrVV-NHis-immunized mice after SARS-CoV infection may be related to the severity of the pulmonary inflammation in these mice. Weingartl et al. (47) and Czub et al. (48) reported that immunization with S protein expressing-recombinant modified VV Ankara (rMVA-S) induced stronger inflammatory responses and focal necrosis in liver tissues after SARS-CoV challenge than in control animals. However, the precise mechanism underlying this liver inflammation has not been clarified. Feline infectious peritonitis virus, which is another member of the coronavirus family, exhibits enhanced infection into monocytes or macrophages through virus-specific Ab binding to the Fc receptors of these cells and causes enhanced inflammation (49). It has also been reported for dengue virus that secondary infection with a different genotype results in more severe symptoms, including dengue hemorrhagic fever and dengue shock syndrome. The exacerbation of this symptom is also positively associated with pre-existing Abs with specificity for dengue virus (50). In the case of SARS-CoV, Ab-dependent enhancement of infection has not been reported previously. We hypothesized that the severe pneumonia observed in mOrVV-NHis-immunized mice after SARS-CoV infection does not result from Ab-dependent enhancement because the virus titers in the mouse lungs 9 days later were below the detection limit. Deming et al. (51) 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. It has also been reported that immunization with formalin-inactivated respiratory syncytial virus vaccine and VV that expresses the G glycoprotein of respiratory syncytial virus correlates with the augmentation of Th2-type immune responses and enhanced pulmonary disease (52, 53). Therefore, 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. (54) have demonstrated that prior immunization with N protein generates stronger Ag-specific Th1 and Th2 responses than immunization with M or E protein. In addition, we demonstrate the suppression of anti-inflammatory cytokine responses in N protein-immunized mice. Interestingly, Shi et al. (55) demonstrated that coinjection of M protein with N protein not only enhanced the production of Th1 cytokines (IFN-γ and IL-2), but also reduced the rates of mortality and pathologic change in SARS-CoV-infected voles. 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. 3*B*). Furthermore, the m8rVV-S-immunized group exhibited alleviation of the pulmonary histopathology, as compared with both control groups after 9 days. To date, various types of SARS vaccine, including recombinant vaccines, inactivated vaccines, and DNA vaccine, have been reported (34–39). There are only a few reports on the effect of a single immunization with recombinant SARS vaccines, namely SARS-CoV S protein-expressing vaccines based on rabies virus (56), vesicular stomatitis virus (39), and adeno-associated virus (57). It is noteworthy that a single i.m. immunization with recombinant adeno-associated virus that expresses the receptor-binding domain of S protein conferred long-term protection against SARS-CoV infection (57). In the present study, we also show that a single immunization with m8rVV-S reduces viral load and improves the histopathologic findings in the lungs of BALB/c

mice infected with high-titer (1×10^5 $TCID_{50}$/body) SARS-CoV, although a relatively low titer of SARS-CoV was used in the previous study conducted by Du et al. (57). 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 summary, 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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Disclosures

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References

1. Drosten, C., S. Gunther, W. Preiser, S. van der Werf, H. R. Brodt, S. Becker, H. Rabenau, M. Panning, L. Kolesnikova, R. A. Fouchier, et al. 2003. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *N. Engl. J. Med.* 348: 1967–1976.
2. Ksiazek, T. G., D. Erdman, C. S. Goldsmith, S. R. Zaki, T. Peret, S. Emery, S. Tong, C. Urbani, J. A. Comer, W. Lim, et al. 2003. A novel coronavirus associated with severe acute respiratory syndrome. *N. Engl. J. Med.* 1953–1966.
3. Peiris, J. S., S. T. Lai, L. L. Poon, Y. Guan, L. Y. Yam, W. Lim, J. Nicholls, W. K. Yee, W. W. Yan, M. T. Cheung, et al. 2003. Coronavirus as a possible cause of severe acute respiratory syndrome. *Lancet* 361: 1319–1325.
4. Rota, P. A., M. S. Oberste, S. S. Monroe, W. A. Nix, R. Campagnoli, J. P. Icenogle, S. Penaranda, B. Bankamp, K. Maher, M. H. Chen, et al. 2003. Characterization of a novel coronavirus associated with severe acute respiratory syndrome. *Science* 300: 1394–1399.
5. Li, W., M. J. Moore, N. Vasilieva, J. Sui, S. K. Wong, M. A. Berne, M. Somasundaran, J. L. Sullivan, K. Luzuriaga, T. C. Greenough, et al. 2003. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature* 426: 450–454.
6. Hamming, I., W. Timens, M. L. Bulthuis, A. T. Lely, G. J. Navis, and H. van Goor. 2004. Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. *J. Pathol.* 203: 631–637.
7. Jones, B. M., E. S. Ma, J. S. Peiris, P. C. Wong, J. C. Ho, B. Lam, K. N. Lai, and K. W. Tsang. 2004. Prolonged disturbances of in vitro cytokine production in patients with severe acute respiratory syndrome (SARS) treated with ribavirin and steroids. *Clin. Exp. Immunol.* 135: 467–473.
8. Wong, C. K., C. W. Lam, A. K. Wu, W. K. Ip, N. L. Lee, I. H. Chan, L. C. Lit, D. S. Hui, M. H. Chan, S. S. Chung, and J. J. Sung. 2004. Plasma inflammatory cytokines and chemokines in severe acute respiratory syndrome. *Clin. Exp. Immunol.* 136: 95–103.
9. Rowe, T., G. Gao, R. J. Hogan, R. G. Crystal, T. G. Voss, R. L. Grant, P. Bell, G. P. Kobinger, N. A. Wivel, and J. M. Wilson. 2004. Macaque model for severe acute respiratory syndrome. *J. Virol.* 78: 11401–11404.
10. Osterhaus, A. D., R. A. Fouchier, and T. Kuiken. 2004. The aetiology of SARS: Koch's postulates fulfilled. *Philos. Trans. R. Soc. Lond B Biol. Sci.* 359: 1081–1082.
11. Subbarao, K., J. McAuliffe, L. Vogel, G. Fahle, S. Fischer, K. Tatti, M. Packard, W. J. Shieh, S. Zaki, and B. Murphy. 2004. Prior infection and passive transfer of neutralizing antibody prevent replication of severe acute respiratory syndrome coronavirus in the respiratory tract of mice. *J. Virol.* 78: 3572–3577.
12. Glass, W. G., K. Subbarao, B. Murphy, and P. M. Murphy. 2004. Mechanisms of host defense following severe acute respiratory syndrome-coronavirus (SARS-CoV) pulmonary infection of mice. *J. Immunol.* 173: 4030–4039.
13. Roberts, A., L. Vogel, J. Guarner, N. Hayes, B. Murphy, S. Zaki, and K. Subbarao. 2005. Severe acute respiratory syndrome coronavirus infection of golden Syrian hamsters. *J. Virol.* 79: 503–511.
14. ter Meulen, J., A. B. Bakker, E. N. van den Brink, G. J. Weverling, B. E. Martina, B. L. Haagmans, T. Kuiken, J. de Kruif, W. Preiser, W. Spaan, et al. 2004. Human monoclonal antibody as prophylaxis for SARS coronavirus infection in ferrets. *Lancet* 363: 2139–2141.
15. Roberts, A., C. Paddock, L. Vogel, E. Butler, S. Zaki, and K. Subbarao. 2005. Aged BALB/c mice as a model for increased severity of severe acute respiratory syndrome in elderly humans. *J. Virol.* 79: 5833–5838.
16. Hong, T. C., Q. L. Mai, D. V. Cuong, M. Parida, H. Minekawa, T. Notomi, F. Hasebe, and K. Morita. 2004. Development and evaluation of a novel loop-mediated isothermal amplification method for rapid detection of severe acute respiratory syndrome coronavirus. *J. Clin. Microbiol.* 42: 1956–1961.
17. Kitabatake, M., S. Inoue, F. Yasui, S. Yokochi, M. Arai, K. Morita, H. Shida, M. Kidokoro, F. Murai, M. Q. Le, K. Mizuno, et al. 2007. SARS-CoV spike protein-expressing recombinant vaccinia virus efficiently induces neutralizing antibodies in rabbits pre-immunized with vaccinia virus. *Vaccine* 25: 630–637.
18. Hsieh, P. K., S. C. Chang, C. C. Huang, T. T. Lee, C. W. Hsiao, Y. H. Kou, I. Y. Chen, C. K. Chang, T. H. Huang, and M. F. Chang. 2005. Assembly of severe acute respiratory syndrome coronavirus RNA packaging signal into virus-like particles is nucleocapsid dependent. *J. Virol.* 79: 13848–13855.
19. Zhang, C. H., J. H. Lu, Y. F. Wang, H. Y. Zheng, S. Xiong, M. Y. Zhang, X. J. Liu, J. X. Li, Z. Y. Wan, X. G. Yan, et al. 2005. Immune responses in BALB/c mice induced by a candidate SARS-CoV inactivated vaccine prepared from F69 strain. *Vaccine* 23: 3196–3201.
20. Biacchesi, S., M. H. Skiadopoulos, L. Yang, B. R. Murphy, P. L. Collins, and U. J. Buchholz. 2005. Rapid human metapneumovirus microneutralization assay based on green fluorescent protein expression. *J. Virol. Methods* 128: 192–197.
21. Cimolai, N., G. P. Taylor, D. Mah, and B. J. Morrison. 1992. Definition and application of a histopathological scoring scheme for an animal model of acute *Mycoplasma pneumoniae* pulmonary infection. *Microbiol. Immunol.* 36: 465–478.
22. Luna, L. G., 1968. *Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology*. McGraw-Hill, New York, p. 111.
23. Voss, D., A. Kern, E. Traggiai, M. Eickmann, K. Stadler, A. Lanzavecchia, and S. Becker. 2006. Characterization of severe acute respiratory syndrome coronavirus membrane protein. *FEBS Lett.* 580: 968–973.
24. Ho, Y., P. H. Lin, C. Y. Liu, S. P. Lee, and Y. C. Chao. 2004. Assembly of human severe acute respiratory syndrome coronavirus-like particles. *Biochem. Biophys. Res. Commun.* 318: 833–838.
25. Huang, Y., Z. Y. Yang, W. P. Kong, and G. J. Nabel. 2004. Generation of synthetic severe acute respiratory syndrome coronavirus pseudoparticles: implications for assembly and vaccine production. *J. Virol.* 78: 12557–12565.
26. Buchholz, U. J., A. Bukreyev, L. Yang, E. W. Lamirande, B. R. Murphy, K. Subbarao, and P. L. Collins. 2004. Contributions of the structural proteins of severe acute respiratory syndrome coronavirus to protective immunity. *Proc. Natl. Acad. Sci. USA* 101: 9804–9809.
27. Kim, T. W., J. H. Lee, C. F. Hung, S. Peng, R. Roden, M. C. Wang, R. Viscidi, Y. C. Tsai, L. He, P. J. Chen, et al. 2004. Generation and characterization of DNA vaccines targeting the nucleocapsid protein of severe acute respiratory syndrome coronavirus. *J. Virol.* 78: 4638–4645.
28. Fan, B. X., L. X. Xie, L. A. Chen, W. J. Chen, J. Wen, and Y. N. Liu. 2005. Study on the dynamics of IgG antibody in 311 patients with severe acute respiratory syndrome. *Zhonghua Liu Xing Bing Xue Za Zhi.* 26: 194–196.
29. Che, X. Y., L. W. Qiu, Z. Y. Liao, Y. D. Wang, K. Wen, Y. X. Pan, W. Hao, Y. B. Mei, V. C. Cheng, and K. Y. Yuen. 2005. Antigenic cross-reactivity between severe acute respiratory syndrome-associated coronavirus and human coronaviruses 229E and OC43. *J. Infect. Dis.* 191: 2033–2037.
30. Cheng, M., C. W. Chan, R. C. Cheung, R. K. Bikkavilli, Q. Zhao, S. W. Au, P. K. Chan, S. S. Lee, G. Cheng, W. K. Ho, and W. T. Cheung. 2005. Cross-reactivity of antibody against SARS-coronavirus nucleocapsid protein with IL-11. *Biochem. Biophys. Res. Commun.* 338: 1654–1660.
31. Liu, S. J., C. H. Leng, S. P. Lien, H. Y. Chi, C. Y. Huang, C. L. Lin, W. C. Lian, C. J. Chen, S. L. Hsieh, and P. Chong. 2006. Immunological characterizations of the nucleocapsid protein based SARS vaccine candidates. *Vaccine* 24: 3100–3108.
32. Zhu, M. S., Y. Pan, H. Q. Chen, Y. Shen, X. C. Wang, Y. J. Sun, and K. H. Tao. 2004. Induction of SARS-nucleoprotein-specific immune response by use of DNA vaccine. *Immunol. Lett.* 92: 237–243.
33. Zhao, J., Q. Huang, W. Wang, Y. Zhang, P. Lv, and X. M. Gao. 2007. Identification and characterization of dominant helper T-cell epitopes in the nucleocapsid protein of severe acute respiratory syndrome coronavirus. *J. Virol.* 81: 6079–6088.
34. Yang, Z. Y., W. P. Kong, Y. Huang, A. Roberts, B. R. Murphy, K. Subbarao, and G. J. Nabel. 2004. A DNA vaccine induces SARS coronavirus neutralization and protective immunity in mice. *Nature* 428: 561–564.
35. Bukreyev, A., E. W. Lamirande, U. J. Buchholz, L. N. Vogel, W. R. Elkins, M. St Claire, B. R. Murphy, K. Subbarao, and P. L. Collins. 2004. Mucosal immunisation of African green monkeys (*Cercopithecus aethiops*) with an attenuated parainfluenza virus expressing the SARS coronavirus spike protein for the prevention of SARS. *Lancet* 363: 2122–2127.
36. Zhi, Y., J. Figueredo, G. P. Kobinger, H. Hagan, R. Calcedo, J. R. Miller, G. Gao, and J. M. Wilson. 2006. Efficacy of severe acute respiratory syndrome vaccine based on a nonhuman primate adenovirus in the presence of immunity against human adenovirus. *Hum. Gene Ther.* 17: 500–506.
37. Bisht, H., A. Roberts, L. Vogel, K. Subbarao, and B. Moss. 2005. Neutralizing antibody and protective immunity to SARS coronavirus infection of mice induced by a soluble recombinant polypeptide containing an N-terminal segment of the spike glycoprotein. *Virology* 334: 160–165.
38. Bisht, H., A. Roberts, L. Vogel, A. Bukreyev, P. L. Collins, B. R. Murphy, K. Subbarao, and B. Moss. 2004. Severe acute respiratory syndrome coronavirus

spike protein expressed by attenuated vaccinia virus protectively immunizes mice. *Proc. Natl. Acad. Sci. USA* 101: 6641–6646.
39. Kapadia, S. U., J. K. Rose, E. Lamirande, L. Vogel, K. Subbarao, and A. Roberts. 2005. Long-term protection from SARS coronavirus infection conferred by a single immunization with an attenuated VSV-based vaccine. *Virology* 340: 174–182.
40. Jiang, Y., J. Xu, C. Zhou, Z. Wu, S. Zhong, J. Liu, W. Luo, T. Chen, Q. Qin, and P. Deng. 2005. Characterization of cytokine/chemokine profiles of severe acute respiratory syndrome. *Am. J. Respir. Crit. Care Med.* 171: 850–857.
41. Franks, T. J., P. Y. Chong, P. Chui, J. R. Galvin, R. M. Lourens, A. H. Reid, E. Selbs, C. P. McEvoy, C. D. Hayden, J. Fukuoka, et al. 2003. Lung pathology of severe acute respiratory syndrome (SARS): a study of 8 autopsy cases from Singapore. *Hum. Pathol.* 34: 743–748.
42. Nicholls, J. M., L. L. Poon, K. C. Lee, W. F. Ng, S. T. Lai, C. Y. Leung, C. M. Chu, P. K. Hui, K. L. Mak, W. Lim, et al. 2003. Lung pathology of fatal severe acute respiratory syndrome. *Lancet* 361: 1773–1778.
43. Ding, Y., H. Wang, H. Shen, Z. Li, J. Geng, H. Han, J. Cai, X. Li, W. Kang, D. Weng, et al. 2003. The clinical pathology of severe acute respiratory syndrome (SARS): a report from China. *J. Pathol.* 200: 282–289.
44. He, L., Y. Ding, Q. Zhang, X. Che, Y. He, H. Shen, H. Wang, Z. Li, L. Zhao, J. Geng, et al. 2006. Expression of elevated levels of pro-inflammatory cytokines in SARS-CoV-infected ACE2+ cells in SARS patients: relation to the acute lung injury and pathogenesis of SARS. *J. Pathol.* 210: 288–297.
45. Zhang, X., K. Wu, D. Wang, X. Yue, D. Song, Y. Zhu, and J. Wu. 2007. Nucleocapsid protein of SARS-CoV activates interleukin-6 expression through cellular transcription factor NF-κB. *Virology* 365: 324–335.
46. Fuss, I. J., M. Boirivant, B. Lacy, and W. Strober. 2002. The interrelated roles of TGF-β and IL-10 in the regulation of experimental colitis. *J. Immunol.* 168: 900–908.
47. Weingartl, H., M. Czub, S. Czub, J. Neufeld, P. Marszal, J. Gren, G. Smith, S. Jones, R. Proulx, Y. Deschambault, et al. 2004. Immunization with modified vaccinia virus Ankara-based recombinant vaccine against severe acute respiratory syndrome is associated with enhanced hepatitis in ferrets. *J. Virol.* 78: 12672–12676.
48. Czub, M., H. Weingartl, S. Czub, R. He, and J. Cao. 2005. Evaluation of modified vaccinia virus Ankara based recombinant SARS vaccine in ferrets. *Vaccine* 23: 2273–2279.
49. Corapi, W. V., C. W. Olsen, and F. W. Scott. 1992. Monoclonal antibody analysis of neutralization and antibody-dependent enhancement of feline infectious peritonitis virus. *J. Virol.* 66: 6695–6705.
50. Halstead, S. B. 1982. Immune enhancement of viral infection. *Prog. Allergy* 31: 301–364.
51. Deming, D., T. Sheahan, M. Heise, B. Yount, N. Davis, A. Sims, M. Suthar, J. Harkema, A. Whitmore, R. Pickles, et al. 2006. Vaccine efficacy in senescent mice challenged with recombinant SARS-CoV bearing epidemic and zoonotic spike variants. *PLoS Med.* 3:e525.
52. Boelen, A., A. Andeweg, J. Kwakkel, W. Lokhorst, T. Bestebroer, J. Dormans, and T. Kimman. 2000. Both immunisation with a formalin-inactivated respiratory syncytial virus (RSV) vaccine and a mock antigen vaccine induce severe lung pathology and a Th2 cytokine profile in RSV-challenged mice. *Vaccine* 19: 982–991.
53. Johnson, T. R., J. E. Johnson, S. R. Roberts, G. W. Wertz, R. A. Parker, and B. S. Graham. 1998. Priming with secreted glycoprotein G of respiratory syncytial virus (RSV) augments interleukin-5 production and tissue eosinophilia after RSV challenge. *J. Virol.* 72: 2871–2880.
54. Jin, H., C. Xiao, Z. Chen, Y. Kang, Y. Ma, K. Zhu, Q. Xie, Y. Tu, Y. Yu, and B. Wang. 2005. Induction of Th1 type response by DNA vaccinations with N, M, and E genes against SARS-CoV in mice. *Biochem. Bbiophys. Res. Commun.* 328: 979–986.
55. Shi, S. Q., J. P. Peng, Y. C. Li, C. Qin, G. D. Liang, L. Xu, Y. Yang, J. L. Wang, and Q. H. Sun. 2006. The expression of membrane protein augments the specific responses induced by SARS-CoV nucleocapsid DNA immunization. *Mol. Immunol.* 43: 1791–1798.
56. Faber, M., E. W. Lamirande, A. Roberts, A. B. Rice, H. Koprowski, B. Dietzschold, and M. J. Schnell. 2005. A single immunization with a rhabdovirus-based vector expressing severe acute respiratory syndrome coronavirus (SARS-CoV) S protein results in the production of high levels of SARS-CoV-neutralizing antibodies. *J. Gen. Virol.* 86: 1435–1440.
57. Du, L., G. Zhao, Y. Lin, H. Sui, C. Chan, S. Ma, Y. He, S. Jiang, C. Wu, K. Y. Yuen, et al. 2008. Intranasal vaccination of recombinant adeno-associated virus encoding receptor-binding domain of severe acute respiratory syndrome coronavirus (SARS-CoV) spike protein induces strong mucosal immune responses and provides long-term protection against SARS-CoV infection. *J. Immunol.* 180: 948–956.

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Research Article

Reference strand-mediated conformation analysis-based typing of multiple alleles in the rhesus macaque MHC class I *Mamu-A* and *Mamu-B* loci

The rhesus macaque exhibits individual differences in susceptibility and resistance to infectious agents such as simian immunodeficiency virus (SIV) under experimental conditions, and these may be genetically determined at least in part by major histocompatibility complex (*MHC*) class I polymorphism. Although the importance of defining *MHC* class I polymorphism is well recognized, development of a generic and comprehensive molecular typing method of *MHC* class I alleles of the rhesus macaque has been hampered because, during the evolution of this species, multiple copies of similar DNA sequences have been generated by duplication events including the coding sequences of *Mamu-A* and *Mamu-B* loci. We report here a newly developed reference strand-mediated conformation analysis (RSCA)-based typing method of multiple *Mamu-A* and *Mamu-B* cDNAs that allowed us to estimate the number of expressed alleles. This technique detected 1–7 *Mamu-A* signals and 2–12 *Mamu-B* signals in a single sample, indicating that the number of functional alleles may vary. By comparing the data from the parents with those from the descendants in the breeding colony, several *MHC* class I haplotypes consisting of variable numbers of functional *Mamu-A* and *Mamu-B* alleles could be assigned.

Keywords:
MHC class I / Polymorphism / Reference strand-mediated conformation analysis / Rhesus macaques
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1 Introduction

Major histocompatibility complex (MHC; HLA in humans) class I molecules are cell surface glycoproteins that present antigenic peptides to CD8-positive T lymphocytes with killing and other functional activities [1, 2]. Because MHC class I molecules encoded by different alleles exhibit different binding properties to peptides, it is necessary to consider MHC polymorphisms when evaluating an antigen or antigenic peptide as a vaccine to induce efficient protective immunity [3]. The rhesus macaque, *Macaca mulatta*, is an established preclinical animal model for studying prevention and/or treatment of human diseases, including acquired immunodeficiency syndrome (AIDS) [4–6]. Orthologs of *HLA-A* and *HLA-B*, MHC class I genes, were identified in the rhesus macaque and designated *Mamu-A* and *Mamu-B*, respectively. These genes, like the HLA genes, are highly polymorphic [7].

Recent studies on the genomic DNA sequence of the rhesus MHC region have demonstrated that there are multiple tandem gene duplications in the class I region [8, 9]. Genomic genes of *Mamu-A* and *Mamu-B* have reached up to 4 and 19, respectively [8], but it is not known how many of them are transcribed. Because of multiple similar sequences,

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Abbreviations: BLCL, B-lymphoblastoid cell line; **FLR**, fluorescence-labeled reference; **RSCA**, reference strand-mediated conformation analysis; **SIV**, simian immunodeficiency virus

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the analysis of *Mamu-A* and *Mamu-B* polymorphisms is much more complicated than that of HLA class I genes. This was the reason that the allele-specific PCR method has been used to analyze the *Mamu* class I loci, but the allele-specific PCR was able to detect only the presence of specific alleles [10, 11]. In the present study, we aimed to develop a novel typing method based on reference strand-mediated conformation analysis (RSCA) that allowed the resolution of multiple similar but different sequences [12, 13]. Our RSCA-based typing system enabled us to discriminate multiple *Mamu-A* and *Mamu-B* alleles and to estimate the number of transcribed alleles. Using this typing system enabled us to assign several haplotypes, *i.e.*, combinations of *Mamu* class I alleles, in a breeding colony. These results demonstrated that the number of expressed *Mamu* alleles is variable in the rhesus macaque population.

2 Materials and methods

2.1 Animals

Peripheral blood samples were collected from more than 150 rhesus macaques, derived from a breeding colony maintained in Japan to establish B-lymphoblastoid cell lines (BLCLs). Founders of the breeding colony originated from Myanmar (Burma) and Laos.

2.2 Amplification and cloning of *Mamu-A* and *Mamu-B* cDNA

Total cellular RNA was isolated from BLCL using TRIzol reagent (Invitrogen). Oligo (dT)-primed cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's recommendations. Full-length cDNAs of *Mamu-A* and *Mamu-B* were amplified by PCR with locus-specific primer pairs; Mamu-A forward (5′-ATGGCGCCCCGAACCCTCCTCCTGG-3′) with Mamu-A reverse (5′-TCACACTTTACAAGCCGTGAGAGA-3′) and Mamu-B forward (5′-ATGGCGCCCCGAACCCTCCTCCTGC-3′) with Mamu-B reverse (5′-TCAAGCCGTGAGAGACACATC-3′), respectively. PCR was carried out in a standard 25 μL mixture containing 10 pmol of each primer, 0.2 mM dNTPs (Pharmacia), 0.5 units of *Taq* DNA Polymerase (TaKaRa) and 2.5 μL of 10 × PCR Buffer (TaKaRa), and consisted of 34 cycles of: 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min after initial incubation at 95°C for 2 min, and final extension at 72°C for 5 min. The RT-PCR products (cDNAs) were cloned into pGEM-T Easy vector (Promega) and introduced in *Escherichia coli* DH10B (Invitrogen) for sequencing analysis.

2.3 cDNA clones

In addition to the cDNA clones obtained in this study, cDNA clones for *Mamu* alleles, A*01, A*02, A*03, A*0402, A*0504, A*0602, A*07, A*08, A*11, B*01, B*03, B*04, B*0702, B*11, B*12, B*20, B*22, B*26, B*27, and B*29 (kindly provided by Dr. David Watkins, University of Wisconsin [7, 14]), were used to evaluate the power of allele discrimination by the RSCA-based typing method.

2.4 RSCA

The sample for RSCA, *i.e.*, the test cDNA fragment, was a 725 bp-long DNA fragment encoding extracellular domains of the MHC class I molecule, and was prepared by two-step PCR as follows: *Mamu* class I cDNAs were amplified by PCR using a primer pair of Mamu forward (5′-ATGGCGCCCCGAACCCTCCTCCTG-3′) and Mamu reverse (5′-TCACACTTTACAAGCCGTGAGAGA-3′) with 25 cycles of: 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min. The PCR products were diluted and a small aliquot was subjected to nested PCR. The PCR condition was the same as above except that the locus-specific primers were used; primer pairs for *Mamu-A* and *Mamu-B* were Mamu-A forward and Mamu-B forward, respectively, in combination with Mamu 4R (5′-CCAGGTCAGTGTGATCTCCG-3′).

Fluorescence-labeled reference (FLR) was prepared from the cDNA clone of Mamu-B*66 (FLR#1, GenBank Accession #AJ844597) and from the cDNA clone of Mamu-B*39 (FLR#2, GenBank Accession #AJ556890) as templates by PCR using a 5′-fluorochrome Cy5-labeled forward primer (5′-[Cy5]ATGGCGCCCCGAACCCTC-3′) and Mamu 4R. The PCR conditions were as described in Section 2.2, except that the annealing temperature was 58°C. To form heteroduplex DNAs between FLR and test cDNA fragments [12, 13], 1 μL of FLR was added to 3–6 μL of test cDNA fragments and the mixture was denatured at 95°C for 4 min, annealed at 55°C for 5 min, and cooled to 15°C for 5 min. Before applying to polyacrylamide gel, 1 μL of 6 × Ficoll loading buffer (containing 18% Ficoll 400, Pharmacia) and electrophoresis markers were added to the sample. The electrophoresis markers (M#1, M#2, M#3, M#4, M#5, M#6) were Cy5-labeled DNA fragments prepared from the Mamu-A*08 cDNA clone by PCR using the 5′-Cy5-labeled primer in combination with different reverse primers. The following reverse primers were used to prepare the markers (product size is in parentheses after the marker name); 4R for M#1 (585 bp), GEMT257R (5′-GGCTTTACACTTTATGCTTCC-3′) for M#2 (1298 bp), GEMT520R (5′-ATGTTCTTTCCTGCGTTATCC-3′) for M#3 (1561 bp), GEMT698R (5′-GCGCACGAGGGAGCTTCC-3′) for M#4 (1739 bp), GEMT941R (5′-GTTACCAGTGGCTGCTGCC-3′) for M#5 (1918 bp), GEMT1554R (5′-CAGGACCACTTCTGCGCTC-3′) for M#6 (2595 bp). These markers were used as external standards, while M#1 and M#6 were used as internal standards.

The heteroduplex DNAs were subjected to 6% Long Ranger gel (Cambrex BioScience Inc.) in 1 × TBE (89 mM Tris-boric acid, 2 mM EDTA-Na_2, pH 9.0) mounted on ALF express II automated sequencing apparatus (Amersham Biosciences) [12, 13] at 30 W constant power for 480 min. Gel temperature was kept at 40°C during electrophoresis and the

running buffer was 1 × TBE. Data were analyzed with ALFwin Fragment Analyzer software (Amersham Biosciences). Relative mobility of fluorescent DNA was calculated automatically and expressed arbitrarily as a dimensionless number equal to the size of double strand DNA with equivalent mobility. Run-to-run variation in relative mobility values did not exceed 3, which corresponds to RSD of 0.0018 (see footnote of Table 1). Measurements of peak heights and peak areas were used for automatic peak detection but not for molecular typing. They did not show sufficient reproducibility and adjustment of threshold for peak detection was required for each run.

Table 1. Mobility of heteroduplexes between *Mamu-A* and *Mamu-B* alleles and FLRs

Allele	Relative mobility of heteroduplex with[a)]		Comments
	FLR1	FLR2	
Known *Mamu-A* alleles			
Mamu-A*01	1242	1151	
Mamu-A*02	1278	1000	
Mamu-A*03	1348	1134	
Mamu-A*0402	1245	1052	
Mamu-A*0504	1063	1056	=Mamu-A*m90120b3
Mamu-A*0602	1224	1103	
Mamu-A*07	1123	1079	
Mamu-A*08	1280	1137	
Mamu-A*11	1051	1160	
Known *Mamu-B* alleles			
Mamu-B*01	1000	1446	
Mamu-B*03	1041	844	
Mamu-B*04	1042	1055	
Mamu-B*05	1119	956	
Mamu-B*0702	852	1200	
Mamu-B*12	984	1043	
Mamu-B*20	1008	1061	
Mamu-B*22	915	1221	
Mamu-B*26	912	921	
Mamu-B*27	1058	918	
Mamu-B*29	1174	1007	
Alleles isolated in the present study			
Mamu-A*m90120a1	974	918	
Mamu-A*m90120b2	1104	1030	
Mamu-A*m90120b3	1061	1054	=Mamu-A*0504
Mamu-A*m90120a4	1273	1115	
Mamu-A*m90120a5	1035	1180	
Mamu-A*m90122c1	1059	966	
Mamu-A*m90122c2	1359	1186	=Mamu-A*m90010e4
Mamu-A*m90010e4	1358	1185	=Mamu-A*m90122c2

a) Mean value rounded to an integer from at least three different gels. Relative mobility value was obtained from a standard curve of the mobility (in bp) of a double-strand DNA ladder subjected to electrophoresis in parallel (implemented in ALFwin Fragment Analyzer software). Relative mobility did not vary by three or more between different gels. If this range of variation (namely, 841–847 for the peak of 844 at the relative mobility, Mamu-B*03 with FLR#2) is taken as 95% confidence interval, RSD can be estimated thus: (3/844) × (1/1.96) = 0.0018.

3 Results

3.1 RSCA of *Mamu-A* and *Mamu-B* alleles using two FLRs

We first analyzed 20 cDNA clones from known *Mamu* alleles (Mamu-A*01, -A*02, -A*03, -A*0402, -A*0504, -A*0602, -A*07, -A*08, -A*11, -B*01, -B*03, -B*04, -B*0702, -B*11, -B*12, -B*20, -B*22, -B*26, -B*27, and -B*29) and eight cDNA clones obtained in this study from three different BLCLs (derived from R90120, R90010, and R208 [a grandson of R90122]) by RSCA using two FLRs, FLR#1 and FLR#2. Relative mobility values are listed in Table 1 and the variation between different gels was negligible, as described in Section 2.4. A Cartesian plot of the mobility values obtained by two different FLRs (Fig. 1A) revealed that the method could discriminate all the analyzed alleles. Two pairs of cDNA clones with indistinguishable mobility values, *i.e.*, not differing by three in mobility value, were found to be identical in nucleotide sequence; Mamu-A*m90120b3 was identical to Mamu-A*0504, and Mamu-A*m90122c2 was identical to Mamu-A*90010e4, even though they were derived from different individuals. These were hidden duplicates by chance, and demonstrate the power of allele typing by the RSCA method.

When RT-PCR products (cDNA samples) from BLCL were analyzed, multiple fluorescent peaks with different mobility and intensity were observed, suggesting that there were multiple alleles of *Mamu-A* and *Mamu-B* with different expression levels at least in BLCL. To take an example, upon analysis of *Mamu-A* of R90120, five peaks were detected (**a**–**e** in Fig. 1B), and of these, peaks **b**, **d**, and **e** were predominant while **a** and **c** were minor. Analysis of different individuals in the colony demonstrated that the number of peaks detected by this method was variable; for *Mamu-A* from as few as one to as many as seven; for *Mamu-B*, from 2 to 12 (data not shown). As the multiple peak signals with variable content could not be explained by a fixed number of gene copies, we next attempted to determine combinations of alleles (haplotypes) that were transmitted together from parents to offspring.

3.2 Determination of MHC class I haplotypes by RSCA

Assignment of alleles to a haplotype was based on the coappearance of peaks between a breeder and its descendants. In the case of breeder R90120 and its offspring (Fig. 2), three *Mamu-A* and four *Mamu-B* alleles found in R90120 were transmitted to descendants #1, #2, #6, and #7, indicating that these *Mamu-A* and *Mamu-B* alleles reside on the same chromosome, and this combination was designated as haplotype-a (hap-a). Alleles carried on this haplotype were

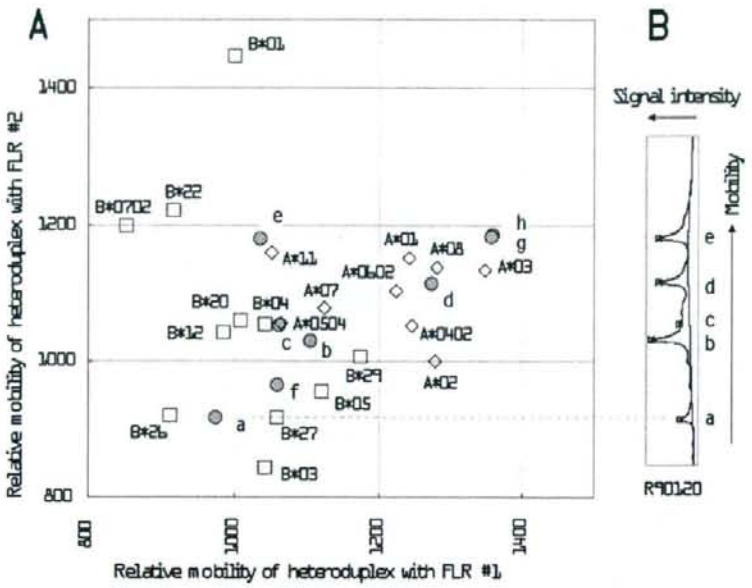

Figure 1. RSCA of *Mamu-A* and *Mamu-B* alleles. (A) Relative mobility of heteroduplex DNA between MHC class I alleles and either FLR#1 (*x*-axis) or FLR#2 (*y*-axis). Alleles isolated in the present study (**a**, Mamu-A*m90120a1; **b**, Mamu-A*m90120b2; **c**, Mamu-A*m90120b3; **d**, Mamu-A*m90120a4; **e**, Mamu-A*m90120a5; **f**, Mamu-A*m90122c1; **g**, Mamu-A*m90122c2; **h**, Mamu-A*m90010e4) are shown as gray circles (●), along with known *Mamu-A* alleles (◇) and *Mamu-B* alleles (□). Note that alleles that could not be distinguished by RSCA, Mamu-A*m90120b3 (**c**) and Mamu-A*0504, as well as Mamu-A*m90122c2 (**g**) and Mamu-A*m90010e4 (**h**), were found to be identical by sequence analysis. (B) Curve view of electrophoresis of heteroduplexes among *Mamu-A* cDNAs derived from R90120 and FLR#2. Peaks indicated by **a** through **e** correspond to respective data points on the plot in panel (A).

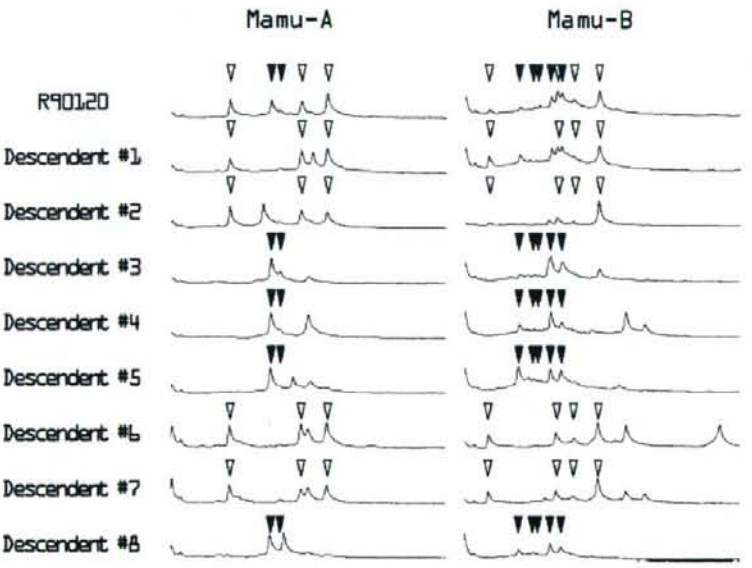

Figure 2. Segregation of *Mamu-A* and *Mamu-B* alleles. A male breeder (R90120) and his eight descendents were analyzed. Three *Mamu-A* and four *Mamu-B* alleles possessed by R90120 (▽) were transmitted to offspring #1, #2, #6, and #7, while the other two *Mamu-A* and five *Mamu-B* alleles (▼) were transmitted to the offspring #3, #4, #5, and #8. This is in accordance with Mendel's law of segregation. Thus, hap-a, which is composed of *Mamu* class I alleles indicated by open triangles, and hap-b, indicated by filled triangles, could be assigned.

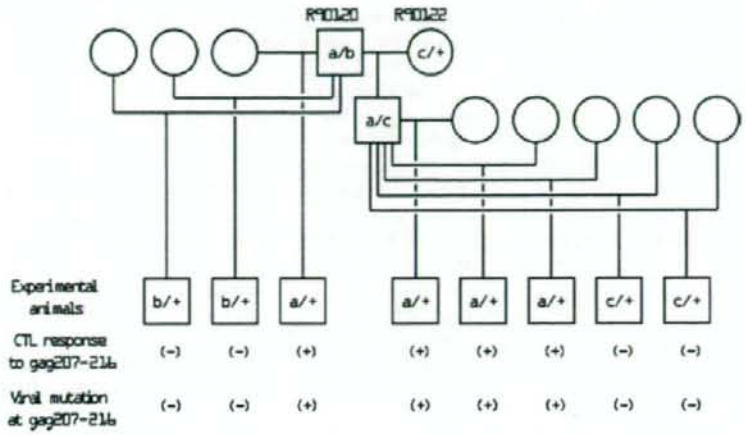

Figure 3. MHC class I haplotype and antiviral response. Induction of cytotoxic T-cell response against SIV gag 207-216 epitope after inoculation of the virus and the emergence of escape mutant (L216S mutation in SIV *gag*) [5] were cosegregated with haplotype a, derived from a male breeder, R90120.

named as Mamu-A*m90120a1, -A*m90120a4, -A*m90120a5, -B*m90120a1, -B*m90120a6, -B*m90120a8, and -B*m90120a9. The other two *Mamu-A* and five *Mamu-B* alleles were transmitted together as haplotype-b (hap-b) to descendants #3, #4, #5, and #8, and were named as A*m90120b2, A*m90120b3, B*m90120b2, B*m90120b3, B*m90120b4, B*m90120b5, and B*m90120b7. As mentioned in the previous section, one of the *Mamu-A* alleles from R90120, A*m90120b3 that exhibited relatively weak expression in BLCL, was found to be identical to A*0504 by nucleotide sequencing.

Similar family studies allowed us to assign another haplotype, hap-c, that was originally possessed by another breeder R90122. Hap-c carried three *Mamu-A* and three *Mamu-B* alleles. Two other haplotypes, d and e, possessed by R90010, carried two *Mamu-A* and two *Mamu-B* alleles and three *Mamu-A* and three *Mamu-B* alleles, respectively. The number of alleles composing each haplotype is summarized in Table 2. Hap-c and hap-e could not be distinguished from each other by RSCA-based typing. Sequencing data of a cDNA clone from each haplotype (Mamu-A*m90122c2 and Mamu-A*90010e4) showed that they were identical, as described in the Section 3.1. Therefore, hap-c and hap-e appeared identical, though it was unclear whether sharing of the same haplotype by two different individuals was due to close kinship or due to the fact that this haplotype was relatively common in the rhesus population in which at least 12 different haplotypes were observed (Table 2).

3.3 Relationship between MHC class I haplotype and epitope-specific T cell response to simian immunodeficiency virus (SIV)

In the rhesus macaque SIV-gag vaccine, gag-specific T cell responses were evoked after challenge by infection with SIV [5]. We observed individual differences among F1 and F2 offspring of R90120 in the induction of cytotoxic T cell response against SIV gag207-216 epitope and in the emergence of escape mutant virus at 216th amino acid residue of gag protein. The phenotype of cytotoxic T lymphocyte response and emergence of escape mutant was completely segregated together with hap-a, suggesting that at least one of the MHC class I alleles carried by hap-a induced antiviral immune response in the vaccine and neither hap-b nor hap-c had com-

Table 2. Number of *Mamu-A* and *Mamu-B* alleles transmitted together as haplotype

Haplotype	Number of MHC class I alleles		Comments
	Mamu-A	*Mamu-B*	
Hap-a	3	4	Possessed by R90120
Hap-b	2	5	Possessed by R90120
Hap-c	3	3	Possessed by R90122[a], (=Hap-e)
Hap-d	2	2	Possessed by R90010
Hap-e	3	3	Possessed by R90010, (=Hap-c)
Hap-f	1	5	Possessed by R90029
Hap-g	3	5	Possessed by R90030
Hap-h	1	2	Possessed by R90030
Hap-j	2	4	Possessed by R90088
Hap-k	4	4	Possessed by R90088
Hap-p	2	4	Possessed by R89002[b]
Hap-q	2	3	Possessed by R89002
Hap-s	3	6	Possessed by R91010[c]
Hap-t	2	4	Possessed by R91010
Hap-u	4	2	Possessed by R96063[c]
Hap-z	3	3	Possessed by R95014[c]

a) R90122 was not analyzed directly but its offspring R208 and others were examined.
b) R89002 originated from Laos.
c) R91010, R96063, and R95014 were breeders born in the colony whose parents originated from Myanmar.

parable immunogenic activities. When the pocket structures of alleles from hap-a were deduced [15, 16], one of the *Mamu-A* alleles, A*m90120a4 (Ala77-Asn80-Leu81-Tyr116), resembled HLA-B*0702 and B*0801 (Ser77-Asn80-Leu81-Tyr116) at F-pocket. This pocket structure showed preference for motifs of aliphatic hydrophobic amino acids as the *C*-terminal anchor residue (Pc), in comparison with the two order difference in binding coefficient values between Leu and Ser. Thus this allele was predicted to be a restriction molecule for SIV gag207-216 epitope, and mutation of Ser in the place of Leu at 216 (Pc anchor site) allowed the virus to evade host immune surveillance [5].

4 Discussion

Rhesus macaques serve as important models for preclinical studies such as developing vaccines for infectious diseases. Characterization of MHC in the experimental animals is required if the epitope-specific response is to be evaluated. It is known that the classical MHC class I loci of the rhesus macaque, *Mamu-A* and *Mamu-B*, exhibit a high degree of allelic variation and complexity due to gene duplication events [7–9]. To date, as many as 42 *Mamu-A* and 81 *Mamu-B* alleles have been isolated from macaques in breeding colonies maintained in North America and Europe [17]. Founders of the colonies originated from India and China, giving rise to the names Indian macaque and Chinese macaque, respectively [18]. In the present study, we analyzed rhesus macaques originating from Myanmar (Burma) or Laos. Only part of our cDNA isolates matched previously reported alleles. This may reflect either the vast sequence variations among the gene pool of the rhesus population or a distant relationship between the original mating groups that diversified due to geographic isolation, as suggested by the mitochondrial DNA sequence divergence [19].

Recently, a method for determining the genotype of MHC class I loci of cynomolgus macaque, *Mafa-A* and *Mafa-B*, by RSCA was published [20], in which the authors developed a typing method using a multicolor DNA sequencer. We also tried to develop a similar method using a multicolor sequencer ABI-377 (Applied Biosystems) in the initial course of the present study, but we found that the sensitivity and S/N were not as good as the ALF sequencer system with Cy5-labeled probes which we then employed here. For example, the signal of Mamu-A*m90120b3 in the R90120 RT-PCR sample was buried by noise level in the ABI system, whereas, it was consistently detected using the ALF sequencer system (peak c of Fig. 1B).

Conventional typing methods were based on PCR using allele-specific oligonucleotide primers. Coverage of detecting *Mamu* alleles by the conventional methods was limited because the sequences available were not sufficient to design primers. In addition, determination of haplotype configuration would have required a laborious procedure of cloning and sequencing alleles from several related individuals. Our typing method, however, was robust enough to find novel alleles, as they can be found simply by measuring difference in mobility on RSCA gel. Haplotype configurations could be assigned easily by comparing the peak view. Another advantage of our method concerns the expression level. Judging by the frequency of isolated cDNA clones, Otting *et al.* [17] suggested an uneven expression of *Mamu-A* alleles belonging to different sublineages. In our system using a single locus-specific primer pair, we observed a considerable difference in signal intensity among alleles comprising the haplotypes shown in Figs. 1 and 2. The difference in intensity may well be correlated with the difference in expression. For example, the smaller peak (labeled as «c» in Fig. 2) encoded by hap-b (identical to Mamu-A*0504) was classified into Mamu-A2 lineage, the alleles of which tend to exhibit lower expression level as compared with alleles of Mamu-A1 lineage [17]. The RSCA-based typing system may therefore have sufficient power to discriminate different sequences among rhesus *MHC* class I alleles, as well as the ability to provide additional information on the relative expression level of the alleles under study.

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5 References

[1] Zinkernagel, R. M., Doherty, P. C., *Nature* 1974, *248*, 701–702.

[2] Rubin, B., Diaz de Durana, Y., Li, N., Sercarz, E. E., *Scand. J. Immunol.* 2003, *57*, 399–409.

[3] Sette, A., Newman, M., Livingston, B., McKinney, D. *et al.*, *Tissue Antigens* 2002, *59*, 443–451.

[4] Desrosiers, R. C., Wyand, M. S., Kodama, T., Ringler, D. J. *et al.*, *Proc. Natl. Acad. Sci. USA* 1989, *86*, 6353–6357.

[5] Matano, T., Kobayashi, M., Igarashi, H., Takeda, A. *et al.*, *J. Exp. Med.* 2004, *199*, 1709–1718.

[6] Mori, K., Sugimoto, C., Ohgimoto, S., Nakayama, E. E. *et al.*, *J. Virol.* 2005, *79*, 10386–10396.

[7] Boyson, J. E., Iwanaga, K. K., Golos, T. G., Watkins, D. I., *J. Immunol.* 1996, *157*, 5428–5437.

[8] Daza-Vamenta, R., Glusman, G., Rowen, L., Guthrie, B., Geraghty, D. E., *Genome Res.* 2004, *14*, 1501–1515.

[9] Kulski, J. K., Anzai, T., Shiina, T., Inoko, H., *Mol. Biol. Evol.* 2004, *21*, 2079–2091.

[10] Knapp, L. A., Lehmann, E., Piekarczyk, M. S., Urvater, J. A., Watkins, D. I., *Tissue Antigens* 1997, *50*, 657–661.

[11] Lobashevsky, A. L., Thomas, J. M., *Hum. Immunol.* 2000, *61*, 1013–1020.

[12] Arguello, J. R., Little, A. M., Pay, A. L., Gallardo, D. *et al.*, *Nat. Genet.* 1998, *18*, 192–194.

[13] Shibata, H., Yasunami, M., Obuchi, N., Takahashi, M. *et al.*, *Hum. Immunol.* 2006, *67*, 47–52.

[14] Urvater, J. A., McAdam, S. N., Loehrke, J. H., Allen, T. M. *et al.*, *Immunogenetics* 2000, *51*, 314–325.

[15] Matsumura, M., Fremont, D. H., Peterson, P. A., Wilson, I. A., *Science* 1992, *257*, 927–934.

[16] Johansen, T. E., McCullough, K., Catipovic, B., Su, X. M. *et al.*, *Scand. J. Immunol.* 1997, *46*, 137–146.

[17] Otting, N., Heijmans, C. M., Noort, R. C., de Groot, N. G. *et al.*, *Proc. Natl. Acad. Sci. USA* 2005, *102*, 1626–1631.

[18] Doxiadis, G. G., Otting, N., de Groot, N. G., de Groot, N. *et al.*, *Immunogenetics* 2003, *55*, 540–551.

[19] Smith, D. G., McDonough, J., *Am. J. Primatol.* 2005, *65*, 1–25.

[20] Krebs, K. C., Jin, Z., Rudersdorf, R., Hughes, A. L., O'Connor, D. H., *J. Immunol.* 2005, *175*, 5230–5239.

