enzyme 2 (ACE2) has been identified as a cellular receptor for SARS-CoV (Li et al., 2003). Thus, the first step of infection likely involves binding of S protein to the ACE2 receptor. In a model of MHV infection, S protein is known to contain important virus-neutralizing epitopes that elicit neutralizing antibody responses in mice (Collins et al., 1982). Therefore, the S protein of coronavirus might be manipulated to induce immunity. However, S, M, and N proteins are also known to contribute to the host immune response (Anton et al., 1996) (Jackwood and Hilt, 1995). A DNA vaccine encoding the S glycoprotein of the SARSCoV induces T cell and neutralizing antibody responses, as well as protective immunity, in a mouse model (Yang et al., 2004). Vaccination with a plasmid expressing N protein is capable of generating strong N-specific humoral and T-cell-mediated immune responses in vaccinated C57BL/6 mice (Kim et al., 2004) (Zhao et al., 2005) (Zhu et al., 2004). In addition, N-specific CD8+ T cells provide protective immunity against some coronaviruses (Collisson et al., 2000; Seo et al., 1997) (Collisson et al., 2000).

The DIs strain is a highly restricted host range mutant of the vaccinia virus isolated by successive one-day egg passage of the DIE vaccinia strain, an authorized strain for smallpox vaccine and actually used in Japan until 1981. DIs does not replicate and is not pathogenic in mice, guinea pigs or rabbits. Furthermore, the DIs does not replicate in various mammalian cell lines (Tagaya, Kitamura, and Sano, 1961). Recently, we established a system for foreign gene expression by inserting target genes into this strain, after which expression of (i) bacteriophage T7 polymerase, and (ii) the full-length HIV-1_{NL432} *gag* gene, was observed (Ishii et al., 2002), thus demonstrating the usefulness of this system.

In the present study, we constructed a recombinant vaccinia virus DIs expressing one or more SARS-CoV structural proteins (E, M, N, and S, or a combination of E, M, and S (E/M/S), or E, M, N and S (E/M/N/S)). These rDIs vaccines were administered to mice either subcutaneously or intranasally, and the humoral and cellular immunity against SARS-CoV in vaccinated mice were analyzed. We demonstrated here that replication-deficient DIs constructs expressing S protein alone or in combination with other components, but not N alone, elicited strong protective immune responses against SARS-CoV infection.

Results

Expression of SARS-CoV structural proteins by rDIs. The structures of transfer vectors used in this study (pDIsSARS-E, pDIsSARS-M, pDIsSARS-N, pDIsSARS-S, pDIsSARS-E/M, pDIsSARS-E/M/S and pDIsSARS-E/M/N/S) were summarized in Fig. 1. Expression of SARS-CoV N and S proteins in chick embryo fibroblast (CEF) cells infected with rDIsSARS was detected by Western blotting using monoclonal antibodies (Fig. 2A) (Ohnishi et al., 2005). Purified SARS-CoV virion was used as a positive control (Fig. 2A, lane PC). A robust signal was detected at 50 kDa, corresponding to the N protein of SARS-CoV, as predicted by its genomic size (Marra et al., 2003) (Rota et al., 2003). A band approaching 200 kDa likely corresponds to the S protein, which is known to be heavily glycosylated (Fig. 2A). Our results are consistent with data reported by Xiao et al. (Xiao et al., 2003) who expressed the full-length S glycoprotein of SARS-CoV Tor2 strain in 293 cells and demonstrated a protein approaching 180-200 kDa by SDS gel electrophoresis. Concerning the M protein, only a smear band in the stacking gel was detected using a polyclonal antibody against synthetic peptide of the M protein (Mizutani et al., 2004), presumably because it formed large oligomers with SDS-resistance in cells (Fig. 2A). Similar result was mentioned by the analysis of the M protein of SARS-CoV (Buchholz et al., 2004) and infectious bronchitis virus (Weisz, Swift, and Machamer, 1993).

The subcellular localization of S, M, and N proteins was analyzed by immunofluorescence staining. Cells infected with rDIsSARS-M demonstrated M proteins primarily co-localized with the Golgi marker GM-130 (Fig. 2B), which is consistent with the results of the recent study (Nal et al., 2005). Individually expressed

SARS-CoV N protein could be detected partially with Golgi apparatus, but remained principally localized to the cytoplasm (Fig. 2B). Overexpressed recombinant SARS-S glycoprotein could be detected partially with Golgi apparatus, but also be detected throughout the cytoplasm (Fig. 2B). These results indicate that cells infected with rDIsSARS expressed significant levels of SARS-CoV proteins under the control of mH5 promoter with an expected post-translational processing (Nal et al., 2005) (You et al., 2005).

rDIsSARS induces serum IgG antibody responses specific for SARS-CoV. To examine the anti-SARS-CoV response in mice after inoculation with rDIsSARS, four mice in each group were subcutaneously or intranasally inoculated three times with 10^6 pfu of rDIsSARS-N, rDIsSARS-M, rDIsSARS-S, rDIsSARS-E/M/S or rDIsSARS-E/M/N/S. Ten days after the final inoculation, vaccinated mice were observed to have high levels of anti-SARS-CoV IgG antibodies in their sera (Fig. 3).

In order to prove effective vaccination, we next examined whether neutralizing antibodies against SARS-CoV were elicited in these mice. As shown in Table 1, neutralizing antibodies against SARS-CoV were induced in mice following subcutaneous or intranasal injection of rDIsSARS-S, rDIsSARS-E/M/S, or rDIsSARS-E/M/N/S, but not in mice immunized with rDIsSARS-N or rDIsSARS-M. These results of ELISA data were incorporated into Fig. 3 by depicting the neutralization positive serum as closed circles. Thus, our results, consistent with others (Bisht et al., 2004; Buchholz et al., 2004) (Yang et al., 2004), indicate that the S protein is a prerequisite for eliciting a sufficient IgG antibody response for neutralization. Similar neutralizing

activity was obtained in mice receiving S alone or in combination with other components. Therefore, we expected that the rDIsSARS expressing E/M/N/S proteins in combination could be the best vaccine candidate among others.

Intranasal inoculation of rDIsSARS expressing E/M/N/S induces SARS-CoV-specific IgA in nasal mucosa and a high level of mucosal IgG in parallel with that of serum IgG

Mucosal IgA response is believed to be crucial for the protective immunity against various pathogens (Meeusen et al., 2004). We, next, examined mucosal immunity in the respiratory tracts of mice inoculated with rDIsSARS either subcutaneously or intranasally. The level of anti-SARS-CoV IgA within nasal wash fluid of vaccinated mice was determined by enzyme-linked immunosorbent assay (ELISA). As shown in Fig. 4A, substantial levels of anti-SARS-CoV IgA were detected only in mice received intranasal inoculation of rDIsSARS-E/M/N/S, compared to those inoculated with parental DIs ($P=0.0010$). The level of IgA detected in intranasally rDIsSARS-E/M/N/S-inoculated mice was similar to that observed following intranasal immunization with UV-inactivated, purified SARS-CoV virion (positive control). On the other hand, subcutaneous injection of all forms of rDIsSARS produced only slightly higher levels of IgA than those observed in DIs -injected control mice. Therefore, the results indicated that the subcutaneous route of injection is inefficient, especially when mucosal IgA response is required.

Since neutralizing activity was, nevertheless, detected in the nasal washes of mice following subcutaneous immunization (data not shown), we also measured anti-SARS-

CoV IgG levels in the nasal washes of these mice (Fig. 4B). High levels of IgG were detected in the nasal washes of mice following nasal immunization, which were observed to correspond well with IgG levels in the serum (Fig. 4C). A similar trend was observed in mice following subcutaneous immunization, despite at a lower level than in mice immunized intranasally. These results suggest that neutralizing IgG antibodies are capable of reaching the mucosal surface if plasma levels are high enough.

Protection of rDIsSARS-immunized mice from nasal SARS-CoV challenge is achieved without mucosal IgA response.

The level of protection against SARS-CoV challenge in mice following inoculation with rDIsSARS is a critical issue for the vaccine development. We inoculated three times with 10^6 pfu of rDIsSARS-N, rDIsSARS-E/M/S or rDIsSARS-E/M/N/S into four mice in each group either subcutaneously or intranasally. One week after final inoculation, the mice were challenged intranasally with 10^4 tissue culture 50% infectious dose ($TCID_{50}$) of SARS-CoV. The results were shown in Fig. 4D. In mice inoculated with saline, 10^3 $TCID_{50}$/ml of SARS-CoV were recovered from lung wash fluid on day 3. In contrast, titers of SARS-CoV from the lungs of mice subcutaneously immunized with rDIsSARS-E/M/S or rDIsSARS-E/M/N/S were below the limits of detection. The same was true for mice intranasally immunized with rDIsSARS-E/M/N/S, whereas the virus was recovered in mice similarly immunized with rDIsSARS-E/M/S. Taken into consideration of a relatively low or marginal level of mucosal IgA antibody in mice intranasally immunized with rDIsSARS-E/M/N/S or rDIsSARS-E/M/S, or even no IgA response by subcutaneous route as described above, it was suggested that mucosal IgG

antibody, but not IgA antibody, likely contributed to the protective immunity, especially in mice simultaneously immunized with recombinant rDIsSARS-E/M/N/S.

On the other hands, titers of SARS-CoV from the lung wash fluid of mice intranasally or subcutaneously immunized with rDIsSARS-N, were similar or slightly lower than the titers of negative controls, suggesting that intranasal or subcutaneous administration of rDIsSARS-N does not protect mice from SARS-CoV challenge, which is highly reflected by the non-neutralizing nature of anti-SARS-CoV N antibodies.

Cellular immunity induced by rDIsSARS.

Although now we know that the systemic neutralizing IgG antibody against SARS-CoV S protein is a major component of protective immunity, T cell responses are also important to protect hosts from various viral infection. In a previous study of coronaviruses, S protein was shown to play an important role in viral pathogenesis, as well as induction of protective immunity (Holmes, 2003). In order to assess the ability of rDIsSARS to induce SARS-CoV S-specific T cells, T cells from axillary lymph nodes (ALN), superficial cervical lymph nodes (CLN) and spleens of mice subcutaneously immunized with rDIsSARS-S or DIs were isolated and stimulated *in vitro* with UV-inactivated, purified SARS-CoV virion. Culture supernatant was collected four days later, and the levels of interferon-γ (IFN-γ), interleukin (IL)-2, IL-4, IL-5 and tumor necrosis factor-α (TNF-α) were measured. T cells in ALN produced the greatest cytokine levels (Fig. 5, and data not shown). This is not surprising in light of the subcutaneous route of immunization. Notably, mice immunized with rDIsSARS-S produced a high level of IFN-γ upon *in vitro* stimulation with UV-inactivated, purified

SARS-CoV virion. The production of TNF-α, an inflammatory cytokine, was significantly elevated in T cells in ALN of rDIsSARS-S immunized mice after *in vitro* stimulation with virion antigens. However, TNF-α production was observed also in mice immunized with parental DIs without *in vitro* stimulation with virion antigens. Since T cells from the lymph nodes of naïve mice did not produce cytokines even after *in vitro* stimulation with virion antigens (data not shown), it is possible that injection of DIs induces mild local inflammation, even when viral proliferation does not occur at the injection site. The pattern of IL-2, IL-5 and IL-4 production were similar to that of IFN-γ, and the maximum level of these cytokines in ALN T cells from rDIsSARS-S-immunized mice were 254, 227 and 88 ng/ml, respectively.

Next, we analyzed the antigenic epitopes of SARS-CoV-specific T cells in the spleen. We carried out IFN-γ enzyme-linked immunospot (ELISPOT) analysis using four 20-mer peptides corresponding to the ACE2 binding region of the S protein selected using the SYFPEITHI score (S44-47), as well as overlapping 20-mer peptides pool covering a whole N protein. When the splenic T cells of mice were analyzed following intranasal or subcutaneous immunization with the most potent vaccine, rDIsSARS-E/M/N/S, a high level of reactivity against S46 was observed especially in the T cells of subcutaneously immunized mice (Fig. 6A). Zhi et al. recently identified a CD4+ T cell epitope, known as NYNYKYRYL, in BALB/c mice (Zhi et al., 2005). S46 contains this sequence, thus, these IFN-γ-producing T cells are likely $CD4^+$ T cells. To detect N-specific T cells, mice subcutaneously or intranasally immunized with rDIsSARS-N were analyzed by ELISPOT. In this case, ten peptides were pooled from amino-terminus of N protein, resulting in 5 pools of peptides. We thus detected N-reactive T cells capable of

recognizing the first 10 peptides pool (Fig. 6B), and observed a greater proportion of N-specific T cells following nasal immunization than subcutaneous immunization. These results indicate that S- and N-specific T cells are generated systemically by rDIs.

In order to elucidate whether or not SARS-CoV-specific $CD8^+$ T cells were induced by immunization with the rDIs, the splenic T cells of mice subcutaneously or intranasally immunized with rDIsSARS-E/M/N/S were further analyzed by ELISPOT using a stably S-expressing A20.2J B cell S6.2 clone, as an antigen presenting cells (APC). Expression of S protein on the S6.2 clone was confirmed by FACS analysis using anti-SARS S monoclonal antibody (Fig. 7A). An empty vector transfectant, BOS-5, was used as a negative control APC. Subcutaneous and intranasal immunization with the most potent rDIsSARS-E/M/N/S generated a significant level of S-specific T cells (Fig. 7B), and a dramatic decrease in S-specific T cells was observed following partial depletion of $CD8^+$ T cells (Fig. 7C). Therefore, rDIsSARS-E/M/N/S was able to induce both SARS-CoV-reactive $CD4^+$ and $CD8^+$ T cells.

Histopathological findings. The immunogenicity of rDIs expressing SARS-CoV structural proteins was further evaluated by histopathological and immunohistochemical analysis of lung tissue in mice, the primary infection site of SARS-CoV (Fig.8). Slight migration of inflammatory cells and mild disruption of the bronchial epithelium were detected in lung tissue of mock-vaccinated mice. SARS-CoV antigens were diffusely observed within the bronchial and alveolar epithelium. In contrast, significant lymphocytic infiltration into peribronchial sites, with little to no detection of SARS-CoV antigens, was observed in mice intranasally immunized with rDIsSARS-E/M/S or

rDIsSARS-E/M/N/S (Fig.8). The infiltrating lymphocytes were found to be CD3-positive T-cells, as determined by immunohistochemistry with anti-CD3 antibody (Fig. 8). On the other hand, intranasal or subcutaneous immunization by only N-expressing DIs induced neither T-cell infiltration nor protective immunity against SARS-CoV, despite of the induction of N-specific antibodies and T cells. These results suggest that marked induction of T-cell response in mice immunized with rDIsSARS-E/M/S and rDIsSARS-E/M/N/S help to eliminate SARS-CoV from the lung tissue. On the other hand, intranasal or subcutaneous immunization by only N-expressing DIs did not induce protective immunity against SARS-CoV, despite of the induction of N-specific antibodies and T cells. Thus, rDIs expressing the S protein, along with other membrane components (E/M and E/M/N), are capable of inducing strong immunity of both humoral and cellular arms and are fully competent to clear SARS-CoV infection.

Discussion

The DIs strain, which replicates well in CEF cells but not in most mammalian cells, was isolated from the DIE strain of the vaccinia virus during serial 1-day egg passage, and it is characterized by the induction of tiny pocks on chicken chorioallantoic membrane (Tagaya, Kitamura, and Sano, 1961) (Kitamura, Kitamura, and Tagaya, 1967) (Ishii et al., 2002). The DIs-derived recombinant viruses express high levels of viral and inserted genes, even in non-permissive cell lines without any cytopathic effects (Ishii et al., 2002). In earlier studies, MVA strain of vaccinia virus, which is also replication-incompetent in most mammal cells, was used to express a variety of foreign genes and some of these recombinant viruses were studied as candidate vaccine vectors and appeared to be more effective than many replication-competent vaccinia virus vaccines (Sutter and Moss, 1992) (Sutter et al., 1994) (Belyakov et al., 1998) (Nam et al., 1999; Sutter and Moss, 1992) (Stittelaar et al., 2000). rDIs does not replicate nor produce infectious virions in most mammalian cells, therefore the DIs strain has a safety advantage when used as a recombinant vaccine vector as for MVA. Recently, a recombinant DIs, rDIsSIVGag, expressing a full-length *gag* gene of SIV, was developed, and demonstrated to have a potential for use as an HIV/AIDS vaccine (Someya et al., 2004).

Attempts at vaccine development against SARS-CoV are ongoing by a number of organizations using various techniques (see review, (Groneberg et al., 2005)). DNA vaccines (Kim et al., 2004) (Yang et al., 2004) (Zhu et al., 2004) (Zhao et al., 2005) and viral vectors such as vaccinia virus (Bisht et al., 2004) (Weingartl et al., 2004), parainfluenza virus (Bukreyev et al., 2004), adenovirus (Zakhartchouk et al., 2005) and

rhabdoviruses (Faber et al., 2005) (Kapadia et al., 2005) are used as recombinant vaccines. Yang *et al.* (Yang et al., 2004) showed that viral replication was reduced by more than six orders of magnitude in the lungs of mice vaccinated with these S plasmid DNA expression vectors, and protection was mediated by a humoral but not a T-cell-dependent immune mechanism. Bisht *et al.* (Bisht et al., 2004) showed that inoculation of BALB/c mice with a recombinant MVA expressing SARS-CoV S protein elicited serum antibodies to SARS-CoV S protein and protective immunity against SARS-CoV infection. Previous studies demonstrated passive transfer of serum from immunized mice conferred protection against SARS-CoV in the respiratory tract following inoculation with either SARS CoV or recombinant MVA expressing S protein (Bisht et al., 2004; Subbarao et al., 2004). These results suggest that S protein is a crucial antigen in generating protective immunity. We observed that intranasal or subcutaneous inoculation of BALB/c mice with rDIs expressing S protein (rDIsSARS-S, rDIsSARS-E/M/S or rDIsSARS-E/M/N/S) produced serum antibodies capable of recognizing the SARS-CoV virion by ELISA, also capable of neutralizing SARS-CoV *in vitro*. The subcutaneous route appears to elicit stronger immunity than intranasal immunization with respect to the level of anti-SARS-CoV IgG antibody produced. Important finding here is that although the mucosal IgA antibody response was induced only in mice intranasally immunized with rDIsSARS-E/M/N/S, the mice administered with rDIsSARS-E/M/N/S by either route elicited strong protective immunity. Therefore, the protection was achieved in the absence of a mucosal IgA response in mice subcutaneously immunized with rDIsSARS-E/M/N/S. Thus, our results clearly show that mucosal infection might be prevented in the presence of a high level of neutralizing

serum IgG antibody. Control mice vaccinated with DIs not expressing envelope proteins were not protected, indicating that the effect was specific for the expressed envelope proteins of SARS-CoV and was not due to enhanced nonspecific immunity.

A recent report suggests that a combination of three adenovirus vector expressing SARS-S, -M and –N protein is capable of eliciting neutralizing antibodies in serum and N-specific T cell responses in rhesus macaques (Gao et al., 2003). However, in this report, the relationship between these immune responses and actual protection was not presented. In another study, the importance of SARS-CoV structural proteins in generating protective immunity was investigated by expressing them individually and in combination using a recombinant parainfluenza virus (PIV) type 3 vector. The expression of S with the two other putative virion envelope proteins, M and E protein, did not augment the neutralizing antibody response. In the absence of S, expression of M and E, or the nucleocapsid protein N, did not induce a detectable serum SARS-CoV-neutralizing antibody response (Buchholz et al., 2004). Our results were consistent with this in that expression of M or N proteins by administration of DIs harboring SARS-CoV M or N gene singly did not induce a neutralizing antibody response, although anti-SARS-CoV antibodies were detected by ELISA.

Recent studies have shown that vaccination with a plasmid expressing N protein can elicit SARS-CoV nucleocapsid-specific humoral and cellular immune responses (Kim et al., 2004) (Zhu et al., 2004) (Zhao et al., 2005). They showed that linkage of N protein to calreticulin, Ca^{2+}-binding protein known to enhance immune response, in a DNA vaccine resulted in the significant enhancement of the humoral and cellular immune responses to N protein in vaccinated mice. They also showed that the N protein-specific

DNA vaccine elicited partial protection against N protein expressing vaccinia virus challenge, however, the efficacy of N-specific cellular immune responses in protection of SARS-CoV infection is not clear. Although we here showed that N protein expression by rDIs was capable of eliciting N-specific humoral and cellular immunity, vaccination with rDIsSARS-N failed to elicit a neutralizing antibody response against SARS-CoV infection *in vitro*, and failed to confer full protection in vaccinated mice against SARS-CoV challenge, suggesting that SARS-CoV N protein-specific antibodies and CTLs were not sufficient to provide full protection against SARS-CoV infection.

Histopathological analysis of lung tissues in the present study revealed a marked lymphocytic infiltration in peribronchial sites in mice immunized intranasally or subcutaneously with recombinant vaccinia virus DIs expressing E/M/S or E/M/N/S. Almost no SARS-CoV antigens were detected in these areas upon immunohistochemical analysis. The infiltrating lymphocytes were shown to be CD3 positive T-cells. This is the first evidence of induction of protective immunity against SARS-CoV associated with marked infiltration of T cells at a SARS-CoV infection site. We were able to detect SARS-CoV S-specific $CD8^+$ T cells in the spleen of these immunized mice by INF-γ ELISPOT. Thus, these results suggest that T cell induction in mice immunized with rDIsSARS-E/M/S and rDIsSARS-E/M/N/S might provide additional help to completely eliminate SARS-CoV in the lung. Of note, Weingartl *et al.* (Weingartl et al., 2004) reported a low level of neutralizing antibody response in ferrets (*Mustela putorius furo*) immunized with a recombinant MVA expressing SARS-CoV S protein. They also showed that more rapid and vigorous neutralizing antibody responses in immunized ferrets, compared to control animals after challenge with SARS-CoV.

However, SARS-CoV infection and spreading in the immunized ferrets is not prevented. Moreover, upon infection with SARS-CoV, strong inflammatory responses were noted in ferrets immunized with either recombinant MVA expressing SARS-CoV S or N protein, suggesting that vaccination with the recombinant MVA expressing SARS-CoV S or N protein may, in some case, lead to enhanced pathology during SARS-CoV infection (Czub et al., 2005; Weingartl et al., 2004). It was also reported that antibodies that neutralized most human SARS-CoV S enhanced entry mediated by the civet virus S, suggesting the possibility that such kind of vaccines might enhance viral infection (Yang et al., 2005). Although we observed no such pathology in rDIs-immunized mice after virus challenge, the potential for widespread tissue damage following administration of SARS-CoV proteins should be carefully investigated. In addition, further studies are required to clarify whether the recruited T-cells indeed play an important role in clearance of SARS-CoV from sites of infection.

In this study, we constructed rDIs containing genes encoding four structural proteins of SARS-CoV that were individually or simultaneously expressed. Intranasal or subcutaneous inoculation of BALB/c 3T3 mice with rDIs expressing S protein with or without other structural proteins elicited a high level of neutralizing antibodies against SARS-CoV and protective immunity, in the lungs of mice after intranasal challenge. Furthermore, both cellular and mucosal immunity against SARS-CoV structural proteins were also induced following administration of the rDIs. Therefore, the replication-deficient DIs strain is a feasible, safe and effective SARS vaccine vector.

Materials and Methods

Cells. CEF cells and Vero E6 cells were grown in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum (FCS), 1% penicillin-streptomycin and L-glutamine (GIBCO BRL/Life Technologies, Gaithersburg, MD). A20.2J murine B cells were maintained in RPMI1640 supplemented with 10% FCS, 50 μM β-mercaptoethanol, L-glutamine and antibiotics.

Plasmid DNA constructs and DNA preparation. cDNA encoding SARS-CoV structural proteins were generated by reverse transcription of SARS-CoV HKU39849 (Accession No. AY278491) using superscriptII (Invitrogen Corp., Carlsbad, CA), followed by amplification using expand high fidelity PCR system (Roche diagnostics), as described previously (Ohnishi et al., 2005). These DNA fragments encoding E, M, N and S proteins were cloned into the vaccinia virus transfer vector pDIsgptmH5, which also harbored E. coli xanthine-guanine phosphoribosyltransferase, under control of a vaccinia virus p7.5 promoter in the cloning site of pUc/DIs (Ishii et al., 2002), to generate pDIsSARS-E, pDIsSARS-M, pDIsSARS-N, and pDIsSARS-S, respectively. To construct transfer vectors to generate rDIs expressing E/M, E/M/S, or E/M/N/S, DNA fragments encoding M, N and S proteins controlled by the mH5 promoter of the vaccinia virus (Wyatt et al., 1996) were inserted into pDIsSARS-E SmaI, NotI and SacI sites, respectively, thus generating pDIsSARS-E/M, pDIsSARS-E/M/S and pDIsSARS-E/M/N/S (Fig.1). A plasmid expressing S driven by the EF-1α promoter was constructed using pEF-BOS-bst (Yoshizawa et al., 2001), and designated pEF-S-bst.

Generation of recombinant vaccinia virus. Recombinant forms of DIs were obtained by hypoxanthine-guanine phosphoribosyltransferase selection (Falkner and Moss, 1988). Monolayers of CEF cells in 6-well plates were pre-incubated with DMEM containing 10% FCS, 25 μg/ml of micophenoic acid (MPA), 250 μg/ml of xanthine and 15 μg/ml of hypoxanthine. Infection was performed onto CEF cells grown in 8 cm dishes with DIs at a multiplicity of infection (moi) of 1.0. Transfection was performed using 20 μg of each DIs transfer vector and Lipofectamine (GIBCO BRL/Life Technologies). rDIs expressing SARS-CoV structural proteins were selected following four consecutive rounds of plaque purification of CEF cells in 6-well plates pre-incubated with DMEM containing 10% FCS, 25 μg/ml of MPA, 250 μg/ml of xanthine, and 15 μg/ml of hypoxanthine. Resultant rDIs expressing SARS-CoV structural proteins were designated as rDIsSARS-E, rDIsSARS-M, rDIsSARS-N, rDIsSARS-S, rDIsSARS-E/M, rDIsSARS-E/M/S and rDIsSARS-E/M/N/S, respectively and subsequently maintained in CEF cells for use in further studies.

Western blot analysis. CEF cells infected with rDIs constructs harboring ORFs of SARS-CoV structural proteins were fractionated by SDS-PAGE under reduced conditions. Purified SARS-CoV virion (0.5 mg) was used as a positive control. The proteins were then transferred to an Immobilon-P PVDF membrane (MILLIPORE, Bedford, MA) and incubated with monoclonal antibodies against N or S proteins (Ohnishi et al., 2005) or polyclonal antibody against M protein (Mizutani et al., 2004). After washing, the membrane was reacted with HRP-conjugated Fab fragment of anti-mouse or rabbit IgG (H+L) (1:20,000, Santa Cruz Biotechnology, Santa Cruz, CA),

followed by visualization of the bands using chemiluminescent reagents (Pierce, Rockford, IL).

Indirect immunofluorescence analysis. CEF cells were infected with rDIs expressing SARS-CoV structural proteins. After 48 hr of incubation, the cells were washed with PBS and fixed with 3 % paraformaldehyde in PBS for 20 min at room temperature. The fixed cells were then permeabilized with 0.2 % Triton X-100 for 3 min at room temperature, and then blocked with a non-fat milk solution, Block Ace (Yukijirushi Co., Tokyo, Japan). The cells were incubated with polyclonal antibodies against M, N or S proteins (Mizutani et al., 2004) for 60 min at 37 °C. The cells were further incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (TAGO, Burlingame, CA) diluted to 1:500 in PBS to detect SARS-CoV M, N or S proteins. To analyze the subcellular localization of SARS structural proteins, anti-GM-130 monoclonal antibody (BD Biosciences, Mississauga, ON, Canada) and rhodamine-conjugated goat anti-mouse IgG (TAGO) were used to stain the Golgi apparatus.

Vaccination. Animal studies were carried out under a protocol approved by the Animal Care and Use Committee of the National Institute of Infectious Diseases, Japan. Five- to six-week-old female BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan) and immunized with 10^6pfu of rDIs, either subcutaneously (s.c.) or intranasally (i.n.). After two and six weeks, identical titers of recombinant virus were re-administered. One week later, the mice were intranasally challenged with 10^4 $TCID_{50}$ of SARS-CoV in 20 μl of saline as previously described (Subbarao et al., 2004). Three

days later, serum, nasal wash fluid and bronchoalveolar wash fluid were collected to measure viral titers and antibodies against SARS-CoV from mice that were sacrificed under anesthesia with chloroform.

Detection of SARS-CoV specific IgA and IgG antibodies. IgA and IgG titers against SARS-CoV were determined by ELISA, as previously described (Takasuka et al., 2004). Briefly, microtiter plates (Dynatech, Chantily, VA) were coated overnight at 4°C with SARS-CoV-infected or mock-infected Vero E6 cell lysate samples previously treated with 1% NP40, followed by UV-inactivation. The plates were blocked with 1% OVA in PBS-0.05% Tween 20, and then incubated with serially diluted sera (1:10-1:25^5) for 1 h at room temperature. The plates were then incubated with either peroxidase-conjugated anti-mouse IgG (1:2000, Zymed, South San Francisco, CA) or IgA (1:2000, Southern Biotechnology, Birmingham, AL) antibody. The plates were washed three times with PBS-Tween at each step. The substrate mixture (o-phenylenediamine (Zymed) and hydrogen peroxide) was added to each well, and the absorbance of each well was read at 490 nm using a model 680 microplate reader (Bio-Rad, Hercules, CA). To provide a standard for IgG detection, serum was obtained from a hyper-immunized mouse and the OD490nm value of 100 ELISA units/ml of standard serum was around three in every assay. Each SARS-CoV-specific IgG titer was calculated by subtracting the optical density of wells coated with non-infected cell lysate from the optical density of wells coated with virus-infected cell lysate. As a positive control to induce SARS-CoV-specific IgA, mice were immunized intranasally with UV-inactivated purified SARS-CoV together with 3μg of poly(I:C) (Ichinohe et al., 2005).

SARS-CoV neutralizing assay. Sera collected from vaccinated mice were inactivated by incubation at 56°C for 30 min. The serially diluted mice sera (up to five-fold) were incubated with 100 $TCID_{50}$ of SARS-CoV for 1 h, then the mixtures were added to a Vero E6 cell culture grown to confluence in 96-well microtiter plates. After 48 h, the cells were fixed with 10 % formaldehyde and stained with crystal violet to visualize the cytopathic effects of the virus (Storch, 2001). Neutralization antibody titers were expressed as the minimal dilution of sera capable of inhibiting viral cytopathic effects.

Analysis of the SARS-CoV-specific T-cell response. CLN, ALN and spleens were obtained from mice one week after their third vaccination. Following preparation of a single cell suspension, T cells were purified using a Pan T cell isolation kit and a magnetic cell sort system (MACS: Miltenyi Biotec, Bergisch Gladbach, Germany). To prepare APC, normal BALB/c mouse splenocytes were depleted of Thy-1^{+} T cells by MACS and irradiated at 2000 cGy. Purified T cells ($1x10^{6}$) were cultured with APC ($5x10^{6}$) in the presence or absence of UV-irradiated, purified SARS-CoV virion at 10 μg/ml. Four days after cultivation, cytokine concentrations within culture supernatant were measured by flow cytometry using a mouse Th1/Th2 cytokine cytometric bead array kit (Becton Dickinson, San Jose, CA).

Generation of a stable S-transfectant. A20.2J murine B cells were transfected with either pEF-S-bst or pEF-BOS-bst by electroporation at 960 μF and 310 V using a GenePulser (BioRad Laboratories, Hercules, CA). After selection using blasticidine S