JOURNAL OF VIROLOGY, May 2004, p. 4638–4645 Vol. 78, No. 9
0022-538X/04/$08.00+0 DOI: 10.1128/JVI.78.9.4638–4645.2004

Generation and Characterization of DNA Vaccines Targeting the Nucleocapsid Protein of Severe Acute Respiratory Syndrome Coronavirus

Tae Woo Kim,[1] Jin Hyup Lee,[1] Chien-Fu Hung,[1] Shiwen Peng,[1] Richard Roden,[1,2] Mei-Cheng Wang,[3] Raphael Viscidi,[4] Ya-Chea Tsai,[1] Liangmei He,[1] Pei-Jer Chen,[5,6] David A. K. Boyd,[1] and T.-C. Wu[1,2,7,8]*

Departments of Pathology,[1] Pediatrics,[4] Oncology,[7] Biostatistics,[3] Obstetrics and Gynecology,[2] and Molecular Microbiology and Immunology,[8] The Johns Hopkins Medical Institutions, Baltimore, Maryland 21205, and Graduate Institute of Clinical Medicine[5] and Hepatitis Research Center,[6] National Taiwan University Hospital, College of Medicine, National Taiwan University, Taipei, Taiwan

Received 17 October 2003/Accepted 22 December 2003

Severe acute respiratory syndrome (SARS) is a serious threat to public health and the economy on a global scale. The SARS coronavirus (SARS-CoV) has been identified as the etiological agent for SARS. Thus, vaccination against SARS-CoV may represent an effective approach to controlling SARS. DNA vaccines are an attractive approach for SARS vaccine development, as they offer many advantages over conventional vaccines, including stability, simplicity, and safety. Our investigators have previously shown that DNA vaccination with antigen linked to calreticulin (CRT) dramatically enhances major histocompatibility complex class I presentation of linked antigen to $CD8^+$ T cells. In this study, we have employed this CRT-based enhancement strategy to create effective DNA vaccines using SARS-CoV nucleocapsid (N) protein as a target antigen. Vaccination with naked CRT/N DNA generated the most potent N-specific humoral and T-cell-mediated immune responses in vaccinated C57BL/6 mice among all of the DNA constructs tested. Furthermore, mice vaccinated with CRT/N DNA were capable of significantly reducing the titer of challenging vaccinia virus expressing the N protein of the SARS virus. These results show that a DNA vaccine encoding CRT linked to a SARS-CoV antigen is capable of generating strong N-specific humoral and cellular immunity and may potentially be useful for control of infection with SARS-CoV.

Severe acute respiratory syndrome (SARS) has become a priority for healthcare agencies around the world because of its communicability, associated mortality, and the potential for pandemic spread. As of 31 July 2003, 8,098 cases had been identified worldwide and 774 people had died, a mortality rate of about 9.6% (World Health Organization statistics [http://www.who.int/csr/sars/country/table 2003_09_23/en/]). SARS has been attributed to infection with a coronavirus (SARS-CoV) (13, 22, 25). Evidence that SARS-CoV is the etiologic agent of SARS was demonstrated by experimental infection of macaques (*Macaca fascicularis*), fulfilling Koch's postulates (15). Knowledge of the structure of SARS-CoV and characterization of its complete RNA genome (23, 27, 28) have provided us with a significant opportunity to develop strategies for the prevention of SARS using vaccines.

Like its CoV relatives, SARS-CoV is a plus-stranded RNA virus with a ~30-kb genome encoding replicase (*rep*) gene products and the structural proteins spike (S), envelope (E), membrane (M), and nucleocapsid (N) (23, 27). S protein is thought to be involved with receptor binding, E protein plays a role in viral assembly, M is important for virus budding, and N protein is associated with viral RNA packaging (for review, see reference 17). Among these proteins, it is unclear which contain useful SARS-CoV-specific T-cell epitopes. N protein has been shown to generate coronavirus-specific $CD8^+$ T cells, albeit in coronaviruses infecting different species (i.e., mouse hepatitis virus and infectious bronchitis virus) and with different tissue tropisms (3, 4, 30, 34, 35). In addition, N-specific $CD8^+$ T cells have been shown to generate protective effects in other coronavirus systems (9, 30). We therefore initially focused on the N protein of the SARS-CoV as the target antigen for our vaccine development.

DNA vaccines are known for their ability to generate both cellular and humoral antigen-specific immunity (for reviews, see references 12, 26, and 31). DNA is relatively stable, and it can be easily prepared and harvested in large quantities. In addition, naked plasmid DNA is relatively safe and therefore can be repeatedly administered as a vaccine (12, 26). However, naked DNA has no cell type specificity. Thus, it is important to find an efficient route for the delivery of DNA vaccines into the appropriate target cells, such as professional antigen-presenting cells (APC). Intradermal administration of DNA vaccines using a gene gun represents a convenient way to deliver DNA vaccines into professional APCs, such as dendritic cells (DCs), in vivo (10). DCs are the most potent professional APCs that prime $CD4^+$ helper and $CD8^+$ killer T cells in vivo (for review, see references 5, 16, and 33). Thus, gene gun delivery of DNA vaccines to DCs has become an important method for enhancing T-cell-mediated immunity against viral infection.

Our investigators have previously used this system to test several intracellular targeting strategies that enhance major

* Corresponding author. Mailing address: Department of Pathology, The Johns Hopkins University School of Medicine, Ross 512H, 720 Rutland Ave., Baltimore, MD 21205. Phone: (410) 614-3899. Fax: (443) 287-4295. E-mail: wutc@jhmi.edu.

histocompatibility complex (MHC) class I and/or class II processing and presentation of antigen (for review, see reference 19). Recently, our investigators have performed a head-to-head comparison of these strategies for their ability to improve DNA vaccine potency. This comparison showed that linkage of antigen to calreticulin (CRT) in a DNA vaccine resulted in the greatest enhancement of the humoral and T-cell-mediated immune responses in vaccinated mice (J. W. Kim, C. F. Hung, J. Juang, L. He, T. W. Kim, D. K. Armstrong, S. I. Pai, C. T. Lin, and T. C. Wu, submitted for publication). CRT is an abundant 46-kDa Ca^{2+}-binding protein located in the endoplasmic reticulum (24) and is considered to be related to the family of heat shock proteins (2, 11). CRT has been shown to associate with peptides delivered into the endoplasmic reticulum by transporters associated with antigen processing (TAP-1 and TAP-2) (32) and with MHC class I-β2m molecules to aid in antigen presentation (29). These data indicate that DNA vaccines employing CRT can potentially enhance antigen-specific immune responses.

Thus, in the present study, we have vaccinated mice with DNA encoding CRT linked to the N protein of the SARS-CoV. Our data indicate that vaccination with CRT/N DNA vaccine is capable of generating strong N-specific humoral and T-cell-mediated immune responses in vaccinated C57BL/6 mice. More importantly, our data show that mice vaccinated with CRT/N DNA are capable of significantly reducing the titer of challenging vaccinia virus expressing the N protein of the SARS virus. Based on these data, we have considered future development of DNA vaccines for SARS-CoV, as well as eventual clinical translation of our vaccine studies.

MATERIALS AND METHODS

Plasmid DNA constructs and DNA preparation. In the present study we used the mammalian expression vector pcDNA3.1/myc-His(-) (Invitrogen, Carlsbad, Calif.) for our DNA vaccine studies. For the generation of pcDNA3-N-myc, the DNA fragment encoding SARS-CoV nucleocapsid was amplified with PCR using a set of primers, 5′-AAAGAATTCATGTCTGATAATGGACCCCAAT C-3′, 5′-TTTGGTACCTGCCTGAGTTGAATCAGCAGA-3′, and pGEX-1-NC-G3 (L. R. Huang, C. M. Chiu, S. H. Yeh, W. H. Huang, P. R. Hsueh, J. Y. Yang, I. J. Su, S. C. Chang, and P.-J. Chen, submitted for publication), as a template. The amplified product was further cloned into the EcoRI/KpnI sites of pcDNA3.1/*myc*-His(-) vector. To generate pcDNA3-CRT-myc, CRT DNA segment was isolated from pcDNA3-CRT (7) and cloned into the XhoI/EcoRI sites of pcDNA3.1/*myc*-His(-). For the generation of pcDNA3-CRT/N-myc, the amplified N DNA was cloned into the EcoRI/KpnI sites of pcDNA3-CRT-myc. The accuracy of these constructs was confirmed by DNA sequencing. The DNA was amplified in *Escherichia coli* DH5α and purified as described previously (6).

Generation of bacterium-derived SARS-CoV N protein. cDNA encoding SARS-CoV nucleocapsid protein was generated by reverse transcription of SARS-CoV TW1 (18) (accession no. YA291451) using Superscript II (Invitrogen) followed by amplification using platinum *Taq* DNA polymerase (Invitrogen) as described elsewhere (L. R. Huang et al., submitted). The oligonucleotide primers for SARS-CoV N protein were 5′-ATGTCTGATAATGGACCCCA-3′ (forward, nucleotides 28120 to 28139) and 5′-TTATGCCTGAGTTGAATCA G-3′ (reversed, nucleotides 29369 to 29388). The DNA fragment encoding N protein was cloned into the pGEX-1 plasmid (Amersham Pharmacia Biotech, Little Chalfont, England) to generate pGEX-1-NC-G3 (L. R. Huang et al., submitted) for recombinant protein expression. *E. coli* BL-21 cells were transformed with pGEX-1 or pGEX-1-NC-G3 plasmids and grown overnight in Luria-Bertani medium containing 50 μg of ampicillin/ml to the mid-log phase. Cells transformed with glutathione *S*-transferase (GST) or GST-N fusion constructs were directly induced with 0.25 mM isopropyl-β-D-thiogalactoside for 3 h at 30°C. Cells were collected by centrifugation and then resuspended in TNE buffer (50 mM Tris [pH 8.0], 0.15 M NaCl, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride), about 1 ml per 25 (optical density at 600 nm) cells. The fusion protein solubility was determined by sonication and centrifugation followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separation of both the supernatant and pellet fractions. In a larger volume of culture (~3 liters), cells were lysed by using a microfluidizer. Lysates prepared from the large batch were incubated with TNE-equilibrated glutathione resin. Bound protein was eluted by 10 mM reduced glutathione in 50 mM Tris (pH 8.0) buffer. The eluted and purified fractions were used for Western blot analysis and as the coating antigen for an enzyme-linked immunosorbent assay (ELISA).

Western blot analysis. The expression of N protein in 293 cells transfected with pcDNA3.1/*myc*-His(-) encoding no insert, CRT, N, or CRT/N DNA was characterized by Western blot analysis. A 20-μg aliquot of DNA was transfected into 5×10^6 293 cells using Lipofectamine 2000 (Life Technologies, Rockville, Md.). Twenty-four hours after transfection, cells were lysed with protein extraction reagent (Pierce, Rockford, Ill.). Equal amounts of protein (50 μg) were loaded and separated by SDS-PAGE using a 10% polyacrylamide gel. For the characterization of bacterium-derived N protein, 1 μg of purified GST-N fusion protein was loaded and separated by SDS-PAGE using a 10% polyacrylamide gel. The gels were electroblotted to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, Calif.). Blots were blocked with phosphate-buffered saline (PBS)–0.05% Tween 20 (TTBS) containing 5% nonfat milk for 2 h at room temperature. Membranes were probed with rabbit anti-GST-N serum (L. R. Huang et al., submitted) at a 1:1,000 dilution in TTBS for 2 h, washed four times with TTBS, and then incubated with goat anti-rabbit immunoglobulin G (IgG) conjugated to horseradish peroxidase (Zymed, South San Francisco, Calif.) at a 1:1,000 dilution in TTBS containing 5% nonfat milk. Membranes were washed four times with TTBS and developed using Hyperfilm-enhanced chemiluminescence (Amersham, Piscataway, N.J.).

Mice. Six- to eight-week-old female C57BL/6 mice were purchased from the National Cancer Institute (Frederick, Md.) and kept in the oncology animal facility of the Johns Hopkins Hospital (Baltimore, Md.). All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals.

DNA vaccination. DNA-coated gold particles were prepared according to a previously described protocol (6). DNA-coated gold particles were delivered to the shaved abdominal regions of mice using a helium-driven gene gun (Bio-Rad) with a discharge pressure of 400 lb/in^2. C57BL/6 mice were immunized with 2 μg of the plasmid encoding no insert, CRT, N, or CRT/N protein. The mice received two boosters with the same dose at a 1-week interval.

ELISA. The presence of SARS-CoV N-specific antibodies in the sera from CRT/N DNA-vaccinated C57BL/6 mice (five per group) was determined by ELISA using microwell plates coated with bacterium-derived recombinant GST-N protein. Purified GST-N protein was diluted to 1 μg/ml with 0.05 M carbonate buffer (pH 9.6), and 0.1 ml/well was added to 96-well microtiter plates. Purified GST protein was used as negative control. The plates were incubated overnight at 4°C, washed with PBS–0.05% Tween 20 (PT), incubated with PT (0.1 ml/well)–2% bovine serum albumin for 60 min at 37°C, and washed again with PT. Serial dilutions of the tested sera were added (0.1 ml/well), and the plates were incubated for 60 min at 37°C. The plates were washed with PT and were incubated with 0.1 ml of alkaline phosphatase-conjugated rabbit anti-mouse antibodies (Zymed)/well for 30 min at 37°C. The plates were washed with PT and incubated with alkaline phosphatase substrate (0.1 ml/well) (according to Sigma instructions) for 60 min at 37°C. Plates were read on a MicroElisa reader at a wavelength of 450 nm. Readings higher than threefold above negative controls were scored as positive reactions.

Intracellular cytokine staining and flow cytometry analysis. In order to assess the ability of our DNA vaccine encoding SARS-CoV N protein to elicit an N-specific $CD8^+$ T-cell response, we sought to identify the MHC class I-restricted cytotoxic T-cell (CTL) epitope of the SARS-CoV N protein. Using the BIMAS database for D^b and K^b peptide binding predictions (http://bimas.cit.nih.gov/molbio/hla_bind/) and the SYFPEITHI database of MHC ligands and peptide motifs (http://syfpeithi.bmi-heidelberg.com/), we analyzed various peptides of 8, 9, or 10 residues and determined their sequences, positions, and scores, and we eventually generated seven potential peptides for our studies (Table 1). We used splenocytes from C57BL/6 mice vaccinated with CRT/N DNA for the characterization of these candidate peptides. Splenocytes were harvested from mice 1 week after the last vaccination. Prior to intracellular cytokine staining, 4×10^6 pooled splenocytes from the vaccinated mice were incubated for 16 h with 1 μg of each candidate peptide/ml for detecting N-specific $CD8^+$ T-cell precursors. Intracellular gamma interferon (IFN-γ) staining and flow cytometry analysis were performed as described previously (6). Flow cytometry analysis was performed on a Becton Dickinson FACScan with CELLQuest software (Becton Dickinson Immunocytometry Systems, Mountain View, Calif.).

To characterize the various DNA vaccines in eliciting an N-specific $CD8^+$ T-cell response, splenocytes from the various vaccinated mice (five per group) were incubated with 1 μg of N peptide/ml (amino acids [aa] 346 to 354; QFKDNVILL) for 16 h. Intracellular IFN-γ staining and flow cytometry analysis were performed as described above.

Generation and characterization of recombinant vaccinia virus. The recombinant vaccinia virus was generated using a protocol similar to that described previously (38). Briefly, the DNA fragment encoding SARS-CoV nucleocapsid was amplified with PCR using a set of primers, 5′-AAAGCATGCATGTCTGA TAATGGACCCCAATC-3′, 5′-TTTGGTACCTTATGCCTGAGTTGAATCA GCAGA-3′, and pGEX-1-NC-G3, as a template. The amplified product was further cloned into SphI/KpnI sites of pSCIIMCS2. This construct was transfected into wild-type vaccinia virus (Vac-WT)-infected CV-1 using Lipofectamine 2000. The recombinant vaccinia viruses were isolated as described previously (38). Plaque-purified recombinant vaccinia viruses were checked for the expression of N protein by flow cytometry analysis, immunofluorescence staining, and Western blot analysis using rabbit anti-GST-N sera (L. R. Huang et al., submitted). For the detection of the expression of SARS-CoV N protein in TK^- cells infected with vaccinia virus encoding N protein (Vac-N) by flow cytometry analysis, the vaccinia virus-infected cells were incubated with rabbit anti-GST-N serum at a 1:100 dilution in 1× Perm (PharMingen, San Diego, Calif.) for 30 min after fixation with Cytofix/Cytoperm (PharMingen), washed four times with 1× PBS, and then incubated with fluorescein isothiocyanate-labeled goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, Pa.) at a 1:1,000 dilution. Western blot analysis was performed as described above.

Vac-WT and Vac-N were amplified by infecting TK^- cells in vitro according to a standard protocol. The titer was determined by plaque assay using BSC-1 cells. The viral stocks were preserved at −70°C prior to vaccination. Before use, the virus was thawed, trypsinized with a 1/10 volume of trypsin-EDTA in a 37°C water bath for 30 min, and diluted with minimal essential medium to a final concentration of 10^8 PFU/ml.

Immunofluorescence staining for N protein expression. Immunofluorescence staining was performed using a protocol similar to what has been described previously (8). Briefly, TK^- cells were cultured in eight-well culture chamber slides (Nalge Nunc Intl., Naperville, Ill.) until they reached 50% confluency. The cells were infected with Vac-N or Vac WT at a multiplicity of infection of 10 to evaluate the expression of N protein. After 24 h of infection, cells were fixed and permeabilized with Cytofix/Cytoperm (Pharmingen) for 30 min. Rabbit anti-N serum was added into the chamber at a dilution of 1:100 and incubated for 30 min. Diluted fluorescein isothiocyanate–goat anti-rabbit IgG (10 μg/ml; Jackson ImmunoResearch Laboratories) was added and incubated for 30 min. The slides were mounted and observed immediately under a fluorescence microscope.

In vivo challenge with recombinant vaccinia virus. For the local challenge experiment, the immunized mice were anesthetized and infected with 2×10^6 PFU of Vac-WT or Vac-N/mouse in 20 μl by intranasal instillation 1 week after the final immunization. For the systemic challenge experiment, the immunized mice were infected with 10^7 PFU of Vac-N/mouse in 100 μl by intravenous injection 1 week after the final immunization. Five mice were used for each vaccinated group. To determine virus titers in lungs, mice were sacrificed 5 days after challenge. Both lungs were harvested, homogenized in 1 ml of minimal essential medium containing 2.5% fetal bovine serum, and subjected to three rounds of freezing and thawing before the titer of virus was determined by plaque assay.

Statistical analysis. All data expressed as means ± the standard errors of the means are from one experiment of at least two experiments performed. Data for intracellular cytokine staining with flow cytometry analysis and in vivo viral challenge experiments were evaluated by analysis of variance. Comparisons between individual data points were made using Student's *t* test.

RESULTS

Characterization of N protein in cells transfected with the various DNA vaccines. In order to characterize the expression of the SARS-CoV N protein in 293 cells transfected with the various DNA constructs, we performed a Western blot analysis using cell lysates derived from DNA-transfected cells. Rabbit anti-GST-N sera were used for Western blot analysis. As shown in Fig. 1, lysate from 293 cells transfected with N DNA revealed a protein band with a size of approximately M_r 48,000 corresponding to N protein in lane 3. Lysate from 293 cells transfected with CRT/N DNA revealed a protein band with a size of approximately M_r 90,000 corresponding to the chimeric CRT/N protein in lane 4. In contrast, N protein was not detected in lysates from 293 cells transfected with plasmid DNA with no insert (lane 1) or CRT DNA (lane 2). Our data indicated that N DNA-transfected cells exhibited levels of N protein expression comparable to that in CRT/N DNA-transfected cells.

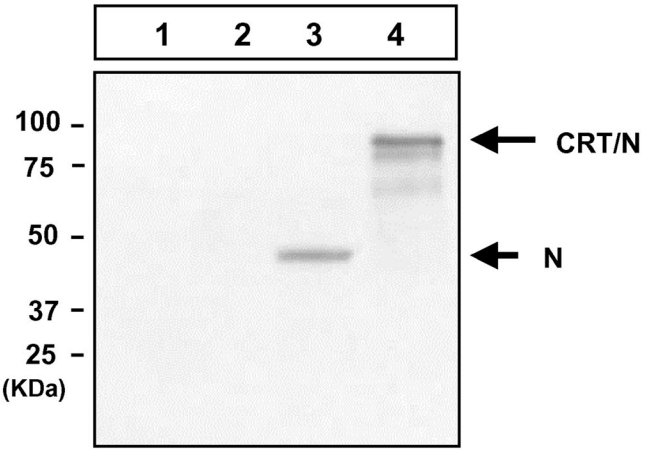

FIG. 1. Characterization of recombinant SARS-CoV N protein expression by Western blot analysis. The expression of SARS-CoV N protein was determined in 293 cells transfected with pcDNA3.1/myc-His(-) encoding CRT, N, CRT/N, or no insert by Western blot analysis. Rabbit anti-GST-N serum was used at a 1:100 dilution for the detection of N expression. Lane 1, lysate from 293 cells transfected with pcDNA3.1/myc-His(-); lane 2, lysate from 293 cells transfected with CRT DNA; lane 3, lysate from 293 cells transfected with N DNA; lane 4, lysate from 293 cells transfected with CRT/N DNA.

Vaccination with CRT/N DNA significantly enhances N-specific antibody responses. To evaluate the humoral immune response to DNA vaccines encoding SARS-CoV N protein, we performed ELISA analysis using bacterium-derived GST-N fusion protein and sera from mice vaccinated with the various DNA vaccines. As shown in Fig. 2A and B, recombinant GST-N protein was purified from bacteria. The purification of bacterium-derived GST-N protein was demonstrated by gel electrophoresis (Fig. 2A). The confirmation of GST-N protein was demonstrated by Western blot analysis with rabbit anti-GST-N sera (Fig. 2B). We used the bacterium-derived GST-N protein for our ELISA. As shown in Fig. 2C, mice vaccinated with CRT/N DNA generated the highest titer of N-specific antibody responses among mice vaccinated with the various DNA vaccines. Furthermore, an ELISA to determine the subtype of IgG antibody showed a significantly higher titer of N-specific IgG1 antibody than N-specific IgG2a in sera from mice vaccinated with N or CRT/N DNA (Fig. 2D). We also used purified GST protein as a control for our ELISA. Sera from vaccinated mice only generated a background level of color changes against GST (data not shown). These data showed that vaccination with CRT/N DNA elicits a significantly stronger N-specific humoral immune response than vaccination with N DNA. This suggests that the linkage of CRT to N protein in a DNA vaccine enhances N-specific antibody production in vaccinated mice.

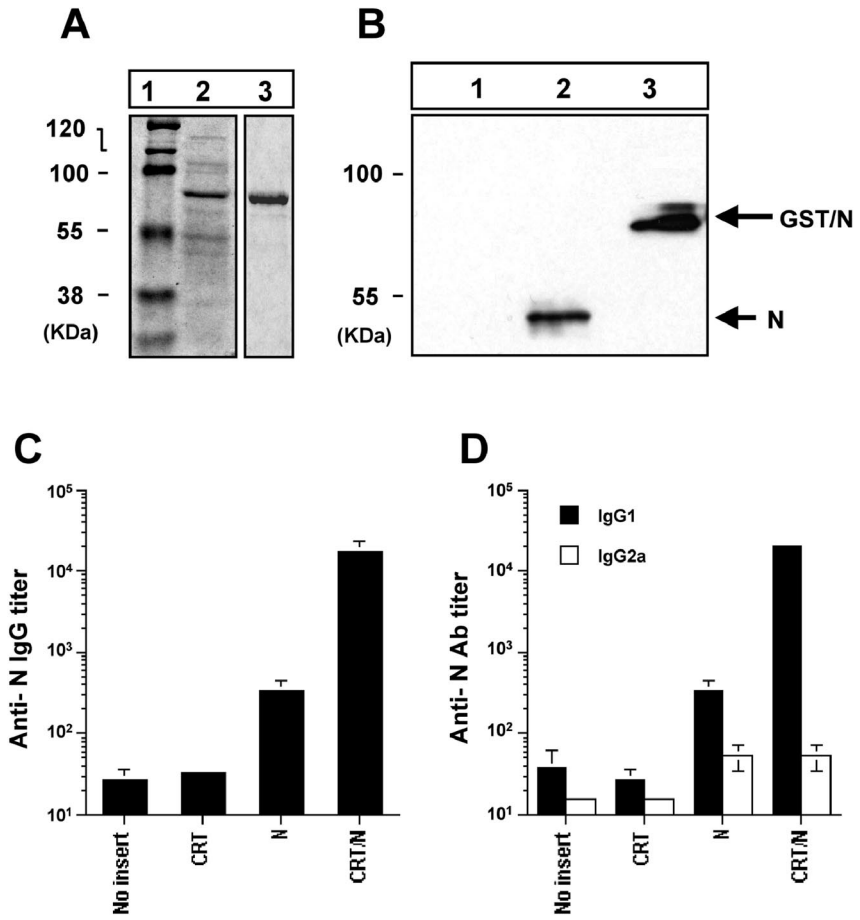

FIG. 2. SARS-CoV N-specific humoral immune response in mice vaccinated with various DNA vaccines. (A) Coomassie blue-stained SDS-PAGE gel of N protein purified from *E. coli*. Lane 1, marker; lane 2, crude extract of *E. coli* expressing N protein; lane 3, purified GST-N protein. (B) Western blot analysis to confirm the presence of purified GST-N protein. Lane 1, lysate from 293 cells transfected with plasmid DNA without an insert (negative control); lane 2, lysate from 293 cells transfected with plasmid DNA encoding N protein (positive control); lane 3, purified GST-N protein. (C) ELISA to determine the titers of N-specific IgG antibodies in sera from vaccinated mice. Sera were collected from DNA-vaccinated mice (five per group) 1 week after the last vaccination and were used to characterize antibodies against bacterium-derived GST-N protein. Purified GST protein was used as a control for our ELISA. Sera from vaccinated mice only generated background levels of color changes against GST (data not shown). (D) ELISA comparing the relative titers of N-specific IgG1 and IgG2a antibodies in sera from DNA-vaccinated mice (five per group).

Vaccination with CRT/N DNA significantly improved SARS-CoV N-specific CD8$^+$ T-cell-mediated immune responses. T-cell-mediated immunity has been shown to be important for control of viral infection. In order to develop quantitative assays for characterizing N-specific CD8$^+$ T-cell-mediated immune responses, we sought to identify the MHC class I-restricted CTL epitope of the SARS-CoV N protein. Using the BIMAS database for D^b and K^b peptide binding predictions (http://bimas.cit.nih.gov/molbio/hla_bind/) and the SYFPEITHIdatabase of MHC ligands and peptide motifs (http://syfpeithi.bmi-heidelberg.com/), we identified several potential candidate peptides for SARS-CoV N protein in

TABLE 1. Candidate CTL epitopes for SARS-CoV nucleocapsid protein

Peptide name	MHC class I	Peptide length	Peptide position	Peptide sequence	BIMAS score	SYFPEITHI score
$N_{346–354}$	*H-2D^b*	9	346–354	QFKDNVILL	60	20
$N_{351–359}$	*H-2D^b*	9	351–359	VILLNKHID	33	11
$N_{352–360}$	*H-2D^b*	9	352–360	ILLNKHIDA	NA[a]	2
$N_{202–211}$	*H-2D^b*	10	202–211	SSRGNSPARM	NA	24
$N_{122–131}$	*H-2D^b*	10	122–131	LPYGANKEGI	200	NA
$N_{50–57}$	*H-2K^b*	8	50–57	TASWFTAL	11	22
$N_{311–318}$	*H-2K^b*	8	311–318	SASAFFGM	11	18

[a] NA, not available.

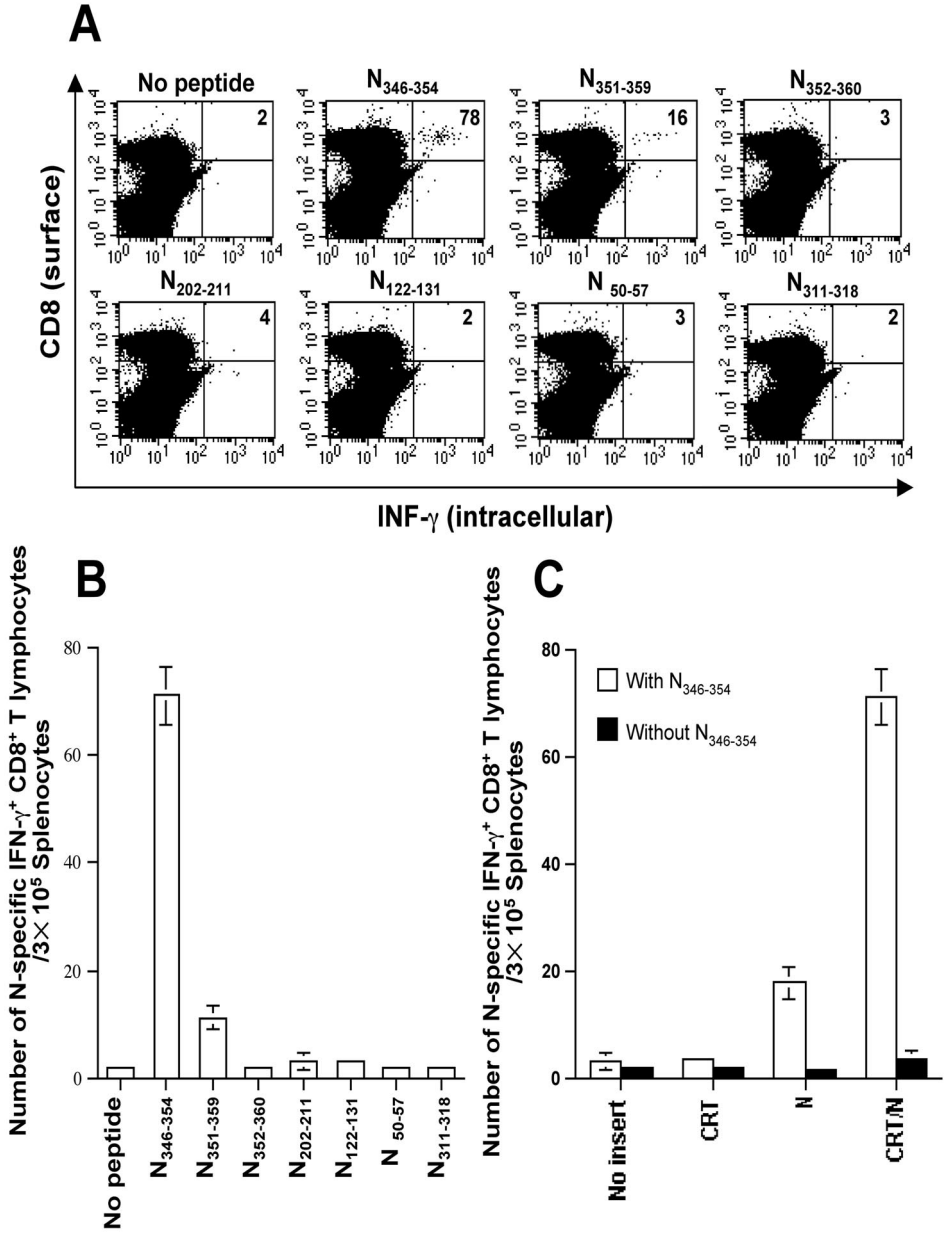

FIG. 3. SARS-CoV N-specific $CD8^+$ T-cell-mediated immune responses in mice vaccinated with the various DNA vaccines. Intracellular cytokine staining followed by flow cytometry analysis was used to characterize the N-specific $CD8^+$ T-cell response to vaccination. (A) Representative example of flow cytometry analysis. (B) Bar graph depicting the number of SARS-CoV N peptide-specific IFN-γ-secreting $CD8^+$ T-cell precursors per 3×10^5 splenocytes. Splenocytes harvested from mice vaccinated with CRT/N DNA (five per group) were incubated with each of the peptides derived from SARS-CoV N protein, as shown in Table 1. (C) Bar graph depicting the number of N-specific IFN-γ-secreting $CD8^+$ T-cell precursors per 3×10^5 splenocytes. Splenocytes from mice (five mice per group) vaccinated with plasmid DNA encoding N, CRT, CRT/N, or no insert were cultured with MHC class I-restricted N peptide (aa 346 to 354; QFKDNVILL) in vitro overnight and stained for CD8 and IFN-γ.

C57BL/6 mice. Table 1 shows their sequences, positions, and scores. We then synthesized these peptides and characterized their ability to activate N-specific $CD8^+$ T cells using splenocytes harvested from mice vaccinated with the various DNA vaccines. As shown in Fig. 3A, using intracellular cytokine staining followed by flow cytometry analysis, we showed that a D^b-restricted 9-mer peptide positioned at aa 346 to 354 (QFKDNVILL) of N protein was able to activate significantly more N-specific $CD8^+$ T cells in splenocytes from mice vaccinated with CRT/N DNA than the other epitopes ($P < 0.05$). In comparison, the N peptide (aa 351 to 359; VILLNKHID) only activated N-specific $CD8^+$ T cells in splenocytes from mice vaccinated with CRT/N DNA to a slightly higher level than the background level. The other five peptides were not able to activate N-specific $CD8^+$ T cells in splenocytes from mice vaccinated with CRT/N DNA (Fig. 3A). Thus, the N peptide (aa 346 to 354; QFKDNVILL) likely represents an $H2D^b$-restricted CTL epitope for SARS-CoV N protein. Our results also showed that mice vaccinated with CRT/N DNA generated significantly more N-specific $CD8^+$ T cells than mice vacci-

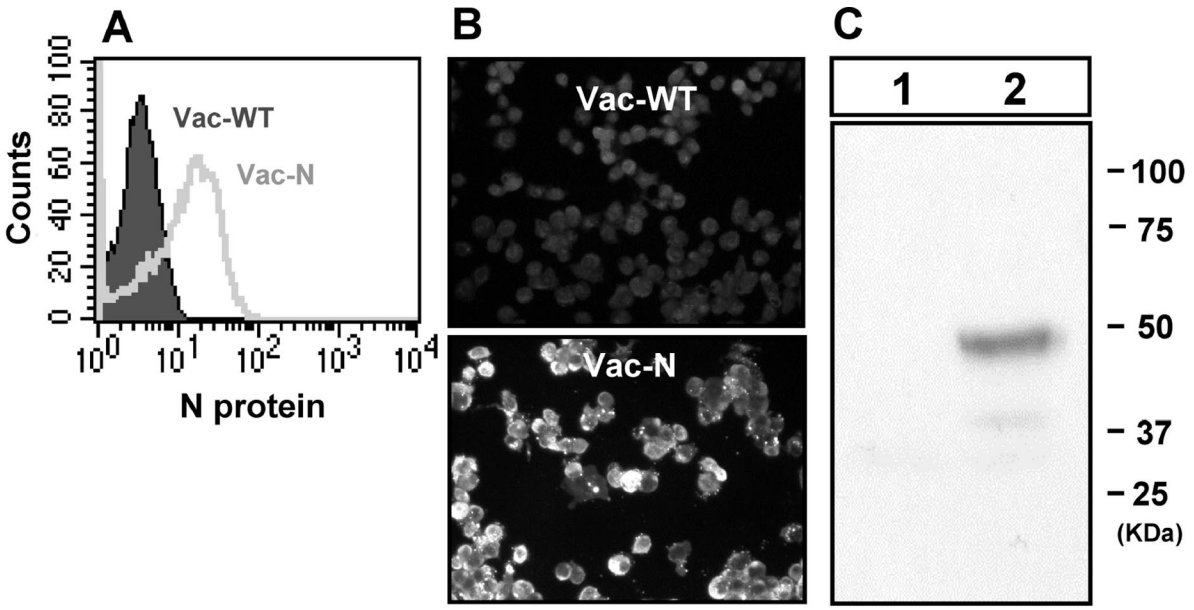

FIG. 4. SARS-CoV N protein expression in cells infected with recombinant N vaccinia virus. 293 cells were infected with either Vac-WT or Vac-N. Rabbit anti-GST-N serum was used to identify N protein expression. (A) Flow cytometry analysis. (B) Immunofluorescence staining. (C) Western blot analysis using cell lysate from 293 cells infected with either Vac-WT (lane 1) or Vac-N (lane 2). Note: lysate from 293 cells infected with Vac-N revealed a band of approximately M_r 48,000 in size, corresponding to the N protein of SARS-CoV.

nated with N DNA (Fig. 3B) ($P < 0.05$). Thus, our data suggest that the linkage of CRT to N protein in a DNA vaccine enhances N-specific $CD8^+$ T-cell-mediated immune responses in vaccinated mice.

Recombinant vaccinia virus expressing SARS-CoV N protein as surrogate virus for vaccine studies. Certain factors preclude the usage of live SARS-CoV for our vaccine efficacy studies. Thus, we generated vaccinia virus expressing SARS-CoV N protein as a surrogate virus for our vaccine efficacy studies. To demonstrate the expression of SARS-CoV N protein, we infected 293 cells with Vac-N and confirmed N expression via flow cytometry analysis, immunofluorescence staining, and Western blot analysis using rabbit anti-GST-N sera (Fig. 4). 293 cells infected with Vac-WT were used as a negative control. All three assays determined that 293 cells infected with Vac-N expressed significant levels of N protein and that 293 cells infected with Vac-WT did not express N protein.

Vaccination with CRT/N DNA results in the greatest reduction of titer of recombinant vaccinia virus expressing N protein. The ability of a vaccine to successfully protect against viral challenge is an essential measure of its efficacy. To test the ability of our DNA vaccines encoding SARS-CoV N protein to protect against viral challenge, we vaccinated mice with DNA encoding CRT/N, N, CRT, or no insert and challenged these mice with Vac-N or Vac-WT intranasally or intravenously 1 week after the last vaccination. As shown in Fig. 5A, while no difference in Vac-WT titer was observed among mice vaccinated with any of the DNA vaccines, we found significantly lower titers of Vac-N in lungs of mice vaccinated with DNA encoding N than in lungs of mice vaccinated with DNA encoding CRT or no insert (intranasal, $P < 0.009$; intravenous, $P < 0.033$). More importantly, mice vaccinated with DNA encoding CRT/N exhibited a significantly reduced titer of Vac-N in their lungs when compared to mice vaccinated with DNA encoding N (intranasal, $P < 0.013$; intravenous, $P < 0.006$). These data indicate that vaccination with CRT/N DNA can reduce the titer of vaccinia virus expressing SARS-CoV N protein to a greater degree than vaccination with N DNA. Thus, vaccination with CRT/N DNA may generate the best protection against intranasal or intravenous challenge with viruses expressing SARS-CoV N protein.

DISCUSSION

We have shown that vaccination with CRT/N DNA can elicit SARS-CoV nucleocapsid-specific humoral and cellular immune responses, and our results suggest that these responses can significantly reduce the titer of challenging vaccinia virus expressing N protein. These results also indicate that the linkage of CRT DNA to N DNA leads to enhanced DNA vaccine potency against a virus expressing a SARS-CoV protein. This is consistent with our previous studies using a different model antigen (human papillomavirus type 16 E7 peptide) (7). Thus, the ability of the CRT strategy to enhance cellular and humoral immune responses has been confirmed in two distinct antigenic systems. This indicates that a similar DNA vaccine strategy may prove effective against other antigenic proteins of SARS-CoV, such as the S, E, or M proteins.

The observed enhancement of the humoral immune response against the N protein of SARS-CoV in mice vaccinated with the chimeric CRT/N DNA vaccine may not be useful for SARS-CoV neutralization, given the location of the N protein inside the viral envelope. Thus, N-specific antibodies may not be able to cross the envelope to bind with the nucleocapsid protein to abolish the infection. In comparison, SARS-CoV S, E, and M proteins are expressed on the envelope surface (27),

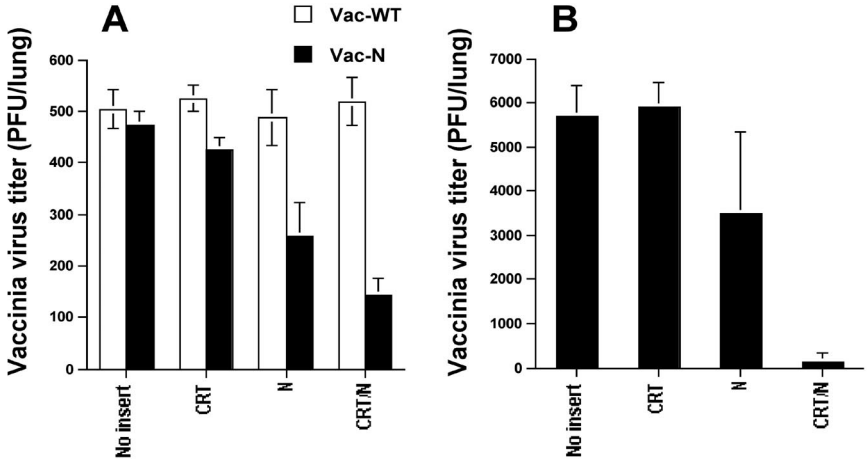

FIG. 5. Reduction of the recombinant N vaccinia virus titer in mice vaccinated with the various DNA vaccines. Mice (five per group) were vaccinated with pcDNA3.1/myc-His(-) encoding CRT, N, CRT/N, or no insert as described in Materials and Methods. (A) Intranasal challenge with vaccinia virus. The immunized mice were infected with 2×10^6 PFU of Vac-WT or Vac-N/mouse in 20 μl by intranasal instillation 1 week after the final immunization. Vac-WT infection was used as a negative control. (B) Intravenous challenge with vaccinia virus. The immunized mice were infected with 10^7 PFU of Vac-N/mouse in 100 μl by intravenous injection 1 week after final immunization. The titer of virus was determined by plaque assay 5 days after challenge. Note: mice vaccinated with CRT/N DNA showed the greatest reduction in titer of Vac-N when challenged intranasally or intravenously.

and neutralizing antibodies against these proteins may thus be able to neutralize SARS-CoV infection. This raises the possibility that a DNA vaccine strategy employing CRT linked to the S, E, or M protein may elicit effective neutralizing antibodies as well as potent T-cell-mediated immune responses against infection by live SARS-CoV. Thus, exploration of the CRT strategy targeting these envelope-associated proteins of SARS-CoV is warranted.

While the humoral immune response may represent an effective means of generating protection from SARS-CoV infection, it may also lead to an antibody-dependent enhancement (ADE) reaction. In ADE, virus-specific antibodies have been shown to interact with the Fc and/or complement receptors to enhance viral entry into host immune cells, such as granulocytic cells and monocyte/macrophages (for review, see reference 36). The ADE phenomenon has been observed in at least one coronavirus system (37). It should therefore be considered when designing a vaccine against SARS-CoV. If the ADE phenomenon is observed in SARS-CoV infection or vaccination, N protein may be the logical choice for a target antigen, as antibodies against N will be unlikely to lead to ADE. This is due to the fact that the N protein is not expressed on the viral envelope and thus antibodies against N will probably not be able to facilitate viral entry.

We observed significant enhancement of the N-specific $CD8^+$ T-cell response as a result of linkage of the N protein to CRT in a DNA vaccine. The percentage of N-specific $CD8^+$ T cells in CRT/N DNA-vaccinated mice may potentially be further improved by coadministration with DNA encoding an antiapoptotic protein (20, 21). Our investigators recently showed that coadministration of DNA encoding BCL-xL with DNA encoding E7/HSP70, CRT/E7, or Sig/E7/LAMP-1 resulted in further enhancement of the E7-specific $CD8^+$ T-cell response for all three constructs (20). Because intracellular targeting and antiapoptotic strategies modify DCs via different mechanisms, it is potentially feasible to combine antiapoptotic strategies for prolonging DC life with CRT for enhancing MHC class I processing and presentation of SARS-CoV antigen by DCs to further enhance DNA vaccine potency.

In this study we used vaccinia virus expressing N protein of SARS-CoV as a surrogate virus for assaying the vaccine efficacy in our study because SARS-CoV, having mainly been isolated in Asia, is difficult to obtain in the United States. More importantly, the handling of live SARS-CoV is potentially extremely hazardous, whereas the handling of recombinant vaccinia virus is relatively safe. For these reasons, we generated vaccinia virus expressing SARS-CoV N protein for use as a surrogate viral challenge model. The development of such a model for testing of our vaccine strategy is not without precedent, as vaccinia virus has been previously used in several prior studies as a substitute viral challenge model (1, 14). While these studies may show a good correlation between the reduction of vaccinia virus titer and vaccine potency, it would be preferable for our research to explore vaccine efficacy against live SARS-CoV in a near-human model. A potential animal model is *M. fascicularis*, which has been shown to be susceptible to live SARS-CoV infection and demonstrates pulmonary pathology similar to humans (15).

We have shown that DNA vaccination can successfully elicit SARS-CoV N-specific humoral and $CD8^+$ T-cell responses in vaccinated mice. It is also evident that vaccination with CRT/N DNA can significantly enhance both humoral and cellular immune responses when compared to vaccination with N DNA. These enhanced immune responses resulting from linkage of antigen to CRT correlate with a strong reduction of titer of challenging vaccinia virus expressing N protein in mice vaccinated with CRT/N DNA. While N protein may not be able to elicit an effective neutralizing antibody response against live SARS-CoV, we have shown that it is capable of eliciting a SARS-CoV antigen-specific $CD8^+$ T-cell response that results in a significant reduction of titer of challenging vaccinia virus when linked to CRT in a DNA vaccine. This makes our CRT/N

DNA vaccine a potential candidate for future clinical translation. Furthermore, our CRT DNA vaccination strategy may potentially be applied to envelope-associated SARS-CoV proteins, such as S, E, or M protein, which may be able to elicit both neutralizing antibodies against SARS-CoV and SARS-CoV antigen-specific CTLs. Future studies will explore the efficacy of DNA vaccines encoding CRT linked to these SARS-CoV structural proteins in a primate model. The results of these studies will help to lay the groundwork for future clinical translation of our DNA vaccine strategy for SARS-CoV.

ACKNOWLEDGMENTS

We thank Robert J. Kurman, Ding-Shinn Cheng, and Ken-Yu Lin for helpful discussions.

This work was supported by a National Institute of Allergy and Infectious Diseases grant (1PO1AI48203-01).

REFERENCES

1. **Arichi, T., T. Saito, M. E. Major, I. M. Belyakov, M. Shirai, V. H. Engelhard, S. M. Feinstone, and J. A. Berzofsky.** 2000. Prophylactic DNA vaccine for hepatitis C virus (HCV) infection: HCV-specific cytotoxic T lymphocyte induction and protection from HCV-recombinant vaccinia infection in an HLA-A2.1 transgenic mouse model. Proc. Natl. Acad. Sci. USA **97:**297–302.
2. **Basu, S., and P. K. Srivastava.** 1999. Calreticulin, a peptide-binding chaperone of the endoplasmic reticulum, elicits tumor- and peptide-specific immunity. J. Exp. Med. **189:**797–802.
3. **Bergmann, C., M. McMillan, and S. Stohlman.** 1993. Characterization of the L^d-restricted cytotoxic T-lymphocyte epitope in the mouse hepatitis virus nucleocapsid protein. J. Virol. **67:**7041–7049.
4. **Boots, A. M., J. G. Kusters, J. M. van Noort, K. A. Zwaagstra, E. Rijke, B. A. van der Zeijst, and E. J. Hensen.** 1991. Localization of a T-cell epitope within the nucleocapsid protein of avian coronavirus. Immunology **74:**8–13.
5. **Cella, M., F. Sallusto, and A. Lanzavecchia.** 1997. Origin, maturation and antigen presenting function of dendritic cells. Curr. Opin. Immunol. **9:**10–16.
6. **Chen, C.-H., T.-L. Wang, C.-F. Hung, Y. Yang, R. A. Young, D. M. Pardoll, and T.-C. Wu.** 2000. Enhancement of DNA vaccine potency by linkage of antigen gene to an HSP70 gene. Cancer Res. **60:**1035–1042.
7. **Cheng, W.-F., C.-F. Hung, C.-Y. Chai, K.-F. Hsu, L. He, M. Ling, and T.-C. Wu.** 2001. Tumor-specific immunity and anti-angiogenesis generated by a DNA vaccine encoding calreticulin linked to a tumor antigen. J. Clin. Investig. **108:**669–678.
8. **Cheng, W. F., C. F. Hung, K. F. Hsu, C. Y. Chai, L. He, J. M. Polo, L. A. Slater, M. Ling, and T. C. Wu.** 2002. Cancer immunotherapy using Sindbis virus replicon particles encoding a VP22-antigen fusion. Hum. Gene Ther. **13:**553–568.
9. **Collisson, E. W., J. Pei, J. Dzielawa, and S. H. Seo.** 2000. Cytotoxic T lymphocytes are critical in the control of infectious bronchitis virus in poultry. Dev. Comp. Immunol. **24:**187–200.
10. **Condon, C., S. C. Watkins, C. M. Celluzzi, K. Thompson, and L. D. Falo, Jr.** 1996. DNA-based immunization by in vivo transfection of dendritic cells. Nat. Med. **2:**1122–1128.
11. **Conway, E. M., L. Liu, B. Nowakowski, M. Steiner-Mosonyi, S. P. Ribeiro, and M. Michalak.** 1995. Heat shock-sensitive expression of calreticulin. In vitro and in vivo up-regulation. J. Biol. Chem. **270:**17011–17016.
12. **Donnelly, J. J., J. B. Ulmer, J. W. Shiver, and M. A. Liu.** 1997. DNA vaccines. Annu. Rev. Immunol. **15:**617–648.
13. **Drosten, C., S. Gunther, W. Preiser, S. van der Werf, H. R. Brodt, S. Becker, H. Rabenau, M. Panning, L. Kolesnikova, R. A. Fouchier, A. Berger, A. M. Burguiere, J. Cinatl, M. Eickmann, N. Escriou, K. Grywna, S. Kramme, J. C. Manuguerra, S. Muller, V. Rickerts, M. Sturmer, S. Vieth, H. D. Klenk, A. D. Osterhaus, H. Schmitz, and H. W. Doerr.** 2003. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N. Engl. J. Med. **348:**1967–1976.
14. **Flory, E., A. Stuhler, V. Barac-Latas, H. Lassmann, and H. Wege.** 1995. Coronavirus-induced encephalomyelitis: balance between protection and immune pathology depends on the immunization schedule with spike protein S. J. Gen. Virol. **76:**873–879.
15. **Fouchier, R. A., T. Kuiken, M. Schutten, G. van Amerongen, G. J. van Doornum, B. G. van den Hoogen, M. Peiris, W. Lim, K. Stohr, and A. D. Osterhaus.** 2003. Aetiology: Koch's postulates fulfilled for SARS virus. Nature **423:**240.
16. **Hart, D. N.** 1997. Dendritic cells: unique leukocyte populations which control the primary immune response. Blood **90:**3245–3287.
17. **Holmes, K. V.** 2003. SARS coronavirus: a new challenge for prevention and therapy. J. Clin. Investig. **111:**1605–1609.
18. **Hsueh, P. R., C. H. Hsiao, S. H. Yeh, W. K. Wang, P. J. Chen, J. T. Wang, S. C. Chang, C. L. Kao, and P. C. Yang.** 2003. Microbiologic characteristics, serologic responses, and clinical manifestations in severe acute respiratory syndrome, Taiwan. Emerg. Infect. Dis. **9:**1163–1167.
19. **Hung, C. F., and T. C. Wu.** 2003. Improving DNA vaccine potency via modification of professional antigen presenting cells. Curr. Opin. Mol. Ther. **5:**20–24.
20. **Kim, T. W., C. F. Hung, D. Boyd, J. Juang, L. He, J. W. Kim, J. M. Hardwick, and T. C. Wu.** 2003. Enhancing DNA vaccine potency by combining a strategy to prolong dendritic cell life with intracellular targeting strategies. J. Immunol. **171:**2970–2976.
21. **Kim, T. W., C. F. Hung, M. Ling, J. Juang, L. He, J. M. Hardwick, S. Kumar, and T. C. Wu.** 2003. Enhancing DNA vaccine potency by coadministration of DNA encoding antiapoptotic proteins. J. Clin. Investig. **112:**109–117.
22. **Ksiazek, T. G., D. Erdman, C. S. Goldsmith, S. R. Zaki, T. Peret, S. Emery, S. Tong, C. Urbani, J. A. Comer, W. Lim, P. E. Rollin, S. F. Dowell, A. E. Ling, C. D. Humphrey, W. J. Shieh, J. Guarner, C. D. Paddock, P. Rota, B. Fields, J. DeRisi, J. Y. Yang, N. Cox, J. M. Hughes, J. W. LeDuc, W. J. Bellini, and L. J. Anderson.** 2003. A novel coronavirus associated with severe acute respiratory syndrome. N. Engl. J. Med. **348:**1953–1966.
23. **Marra, M. A., S. J. Jones, C. R. Astell, R. A. Holt, A. Brooks-Wilson, Y. S. Butterfield, J. Khattra, J. K. Asano, S. A. Barber, S. Y. Chan, A. Cloutier, S. M. Coughlin, D. Freeman, N. Girn, O. L. Griffith, S. R. Leach, M. Mayo, H. McDonald, S. B. Montgomery, P. K. Pandoh, A. S. Petrescu, A. G. Robertson, J. E. Schein, A. Siddiqui, D. E. Smailus, J. M. Stott, G. S. Yang, F. Plummer, A. Andonov, H. Artsob, N. Bastien, K. Bernard, T. F. Booth, D. Bowness, M. Czub, M. Drebot, L. Fernando, R. Flick, M. Garbutt, M. Gray, A. Grolla, S. Jones, H. Feldmann, A. Meyers, A. Kabani, Y. Li, S. Normand, U. Stroher, G. A. Tipples, S. Tyler, R. Vogrig, D. Ward, B. Watson, R. C. Brunham, M. Krajden, M. Petric, D. M. Skowronski, C. Upton, and R. L. Roper.** 2003. The genome sequence of the SARS-associated coronavirus. Science **300:**1399–1404.
24. **Nash, P. D., M. Opas, and M. Michalak.** 1994. Calreticulin: not just another calcium-binding protein. Mol. Cell. Biochem. **135:**71–78.
25. **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, V. C. Cheng, K. H. Chan, D. N. Tsang, R. W. Yung, T. K. Ng, and K. Y. Yuen.** 2003. Coronavirus as a possible cause of severe acute respiratory syndrome. Lancet **361:**1319–1325.
26. **Robinson, H. L.** 1997. Nucleic acid vaccines: an overview. Vaccine **15:**785–787.
27. **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, S. Tong, A. Tamin, L. Lowe, M. Frace, J. L. DeRisi, Q. Chen, D. Wang, D. D. Erdman, T. C. Peret, C. Burns, T. G. Ksiazek, P. E. Rollin, A. Sanchez, S. Liffick, B. Holloway, J. Limor, K. McCaustland, M. Olsen-Rasmussen, R. Fouchier, S. Gunther, A. D. Osterhaus, C. Drosten, M. A. Pallansch, L. J. Anderson, and W. J. Bellini.** 2003. Characterization of a novel coronavirus associated with severe acute respiratory syndrome. Science **300:**1394–1399.
28. **Ruan, Y. J., C. L. Wei, A. L. Ee, V. B. Vega, H. Thoreau, S. T. Su, J. M. Chia, P. Ng, K. P. Chiu, L. Lim, T. Zhang, C. K. Peng, E. O. Lin, N. M. Lee, S. L. Yee, L. F. Ng, R. E. Chee, L. W. Stanton, P. M. Long, and E. T. Liu.** 2003. Comparative full-length genome sequence analysis of 14 SARS coronavirus isolates and common mutations associated with putative origins of infection. Lancet **361:**1779–1785.
29. **Sadasivan, B., P. J. Lehner, B. Ortmann, T. Spies, and P. Cresswell.** 1996. Roles for calreticulin and a novel glycoprotein, tapasin, in the interaction of MHC class I molecules with TAP. Immunity **5:**103–114.
30. **Seo, S. H., L. Wang, R. Smith, and E. W. Collisson.** 1997. The carboxyl-terminal 120-residue polypeptide of infectious bronchitis virus nucleocapsid induces cytotoxic T lymphocytes and protects chickens from acute infection. J. Virol. **71:**7889–7894.
31. **Sin, J. I., and D. B. Weiner.** 2000. Improving DNA vaccines targeting viral infection. Intervirology **43:**233–246.
32. **Spee, P., and J. Neefjes.** 1997. TAP-translocated peptides specifically bind proteins in the endoplasmic reticulum, including gp96, protein disulfide isomerase and calreticulin. Eur. J. Immunol. **27:**2441–2449.
33. **Steinman, R. M.** 1991. The dendritic cell system and its role in immunogenicity. Annu. Rev. Immunol. **9:**271–296.
34. **Stohlman, S. A., S. Kyuwa, M. Cohen, C. Bergmann, J. M. Polo, J. Yeh, R. Anthony, and J. G. Keck.** 1992. Mouse hepatitis virus nucleocapsid protein-specific cytotoxic T lymphocytes are Ld restricted and specific for the carboxy terminus. Virology **189:**217–224.
35. **Stohlman, S. A., S. Kyuwa, J. M. Polo, D. Brady, M. M. Lai, and C. C. Bergmann.** 1993. Characterization of mouse hepatitis virus-specific cytotoxic T cells derived from the central nervous system of mice infected with the JHM strain. J. Virol. **67:**7050–7059.
36. **Tirado, S. M., and K. J. Yoon.** 2003. Antibody-dependent enhancement of virus infection and disease. Viral Immunol. **16:**69–86.
37. **Weiss, R. C., and F. W. Scott.** 1981. Antibody-mediated enhancement of disease in feline infectious peritonitis: comparisons with dengue hemorrhagic fever. Comp. Immunol. Microbiol. Infect. Dis. **4:**175–189.
38. **Wu, T.-C., F. G. Guarnieri, K. F. Staveley-O'Carroll, R. P. Viscidi, H. I. Levitsky, L. Hedrick, K. R. Cho, T. August, and D. M. Pardoll.** 1995. Engineering an intracellular pathway for MHC class II presentation of HPV-16 E7. Proc. Natl. Acad. Sci. USA **92:**11671–11675.