Available online at www.sciencedirect.com

SCIENCE DIRECT

Virology 335 (2005) 34 – 45

VIROLOGY

www.elsevier.com/locate/yviro

Identification of murine CD8 T cell epitopes in codon-optimized SARS-associated coronavirus spike protein

Yan Zhi[a], Gary P. Kobinger[a], Heather Jordan[a], Katie Suchma[b], Susan R. Weiss[b], Hao Shen[b], Gregory Schumer[a], Guangping Gao[a], Julie L. Boyer[c], Ronald G. Crystal[c], James M. Wilson[a,*]

[a]*Gene Therapy Program, Division of Medical Genetics, Department of Medicine, University of Pennsylvania Health System, and The Wistar Institute, Philadelphia, PA 19104, USA*

[b]*Department of Microbiology, School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA*

[c]*Department of Genetic Medicine, Weill Medical College of Cornell University, New York, NY 10021, USA*

Received 16 August 2004; returned to author for revision 19 November 2004; accepted 26 January 2005

Abstract

The causative agent of severe acute respiratory syndrome (SARS) has been identified as a new type of coronavirus, SARS-associated coronavirus (SARS-CoV). CD8 T cells play an important role in controlling diseases caused by other coronaviruses and in mediating vaccine-induced protective immunity in corresponding animal models. The spike protein, a main surface antigen of SARS-CoV, is one of the most important antigen candidates for vaccine design. Overlapping peptides were used to identify major histocompatibility complex class I-restricted epitopes in mice immunized with vectors encoding codon-optimized SARS-CoV spike protein. CD8 T-cell responses were mapped to two H-2^b-restricted epitopes (S436–443 and S525–532) and one H-2^d-restricted epitope (S366–374). The identification of these epitopes will facilitate the evaluation of vaccine strategies in murine models of SARS-CoV infection. Furthermore, codon and promoter optimizations can greatly enhance the overall immunogenicity of spike protein in the context of replication-defective human and simian adenoviral vaccine carriers. The optimized recombinant adenoviral vaccine vectors encoding spike can generate robust antigen-specific cellular immunity in mice and may potentially be useful for control of SARS-CoV infection.

Keywords: SARS-CoV; CD8 T cells; MHC class I epitope; Codon optimization; Promoter optimization; Replication-defective adenoviral vaccine vectors

Introduction

Severe acute respiratory syndrome (SARS) is an emerging infectious disease associated with a novel coronavirus, SARS-associated coronavirus (SARS-CoV), which caused worldwide outbreaks. The case fatality rate has been as high as 15% for patients younger than 60 years old and can be higher than 50% for patients 60 years or older. Nearly 40% of patients developed respiratory failure that required assisted ventilation (De Groot, 2003). The severe morbidity and mortality associated with SARS make it imperative that effective means to prevent and treat the disease be developed and evaluated, especially since it is not known whether the virus will exhibit a seasonal pattern or whether it will be reintroduced into the human population through animal reservoirs, or laboratory accidences, or acts of terrorism.

The successful development of effective treatments and vaccines against SARS-CoV depends on understanding the roles of various immune effectors in protective immunity and identifying protective antigens recognized by these effector cells. Like other CoVs, SARS-CoV is an enveloped plus-stranded RNA virus with a ~30 kbs genome encoding replicase (Rep) gene products and the structural proteins spike (S), envelope (E), membrane (M), and nucleocapsid (N) (Marra et al., 2003; Rota et al., 2003). S protein is responsible for binding to specific cellular receptor (Li et al., 2003), E protein plays a role in viral assembly, M is

* Corresponding author. 125 S 31st Street, TRL, Suite 2000, Philadelphia, PA 19104-3423, USA. Fax: +1 215 898 6588.

E-mail address: wilsonjm@mail.med.upenn.edu (J.M. Wilson).

0042-6822/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2005.01.050

important for virus budding, and N protein is associated with viral RNA packaging (Holmes, 2003). No conclusive information is available on the immune correlates of protection to SARS in patients. However, it has been reported that antibodies against SARS-CoV were detected in patients infected with SARS (Ksiazek et al., 2003). Recently, using pseudotyped lentiviral particles bearing the SARS-CoV spike protein, it has been shown that spike-mediated infection could be inhibited by sera from SARS patients, demonstrating that spike is a target for neutralizing antibodies (Hofmann et al., 2004). In addition, two identified CD8 T-cell epitopes in SARS-CoV spike protein have been shown to elicit specific T-cell responses in HLA-A2+ SARS-CoV-infected patients (Wang et al., 2004). Therefore, we initially focused on SARS-CoV spike protein as the target antigen for our vaccine development.

A number of animal models have emerged for studying SARS pathogenesis and evaluating therapies including macaques, ferrets, and mice (Fouchier et al., 2003; Hogan et al., 2004; Kuiken et al., 2003; Martina et al., 2003; Subbarao et al., 2004). However, for the early stage of product evaluation, a small animal model, such as mice, would be very useful. We (Hogan et al., 2004) and others (Subbarao et al., 2004) have shown that SARS-CoV replicates in mice although the infection is self-limited. To study antigen-specific immune responses in mice, MHC I-restricted CD8 T-cell epitopes need to be identified. Vectors based on replication-defective adenoviruses are capable of high-level gene transfer and activation of T and B cells to the transgene product (van Ginkel et al., 1997; Yang et al., 1996). These properties have been exploited in the development of genetic subunit vaccines (Sullivan et al., 2000; Xiang et al., 1996). Therefore, several versions of replication-defective adenoviral vectors expressing spike protein were created to induce spike-specific T cell responses in mice and to screen for CD8 T-cell epitopes using an overlapping peptide library spanning the entire spike protein in IFN-γ ELISPOT and intracellular IFN-γ staining assays. Here, we report the detailed mapping of both H-2^b- and H-2^d-restricted CD8 T-cell epitopes from codon-optimized spike protein. These results provide critical information for analyzing CD8 T-cell responses in murine models of SARS-CoV infection and for developing spike-based SARS-CoV vaccines. More importantly, a single administration of the optimized SARS-CoV spike vaccine vectors based on replication-defective human and simian adenovirus can generate strong spike-specific CD8 T-cell responses in mice.

Results

Mapping of H-2^b-restricted CD8 T-cell epitopes in SARS-CoV spike protein

To identify CD8 T-cell epitopes in SARS-CoV spike protein, C57BL/6 mice were injected im with AdHu5.CMVSpike, a replication-defective human adenovirus serotype 5-based vaccine vector encoding wild-type spike protein. Splenocytes were harvested 8 days after immunization and stimulated in vitro with pools of overlapping peptides corresponding to spike protein. No pool resulted in specific stimulation of an IFN-γ response in T cells using ELISPOT assay (data not shown).

We then used AdHu5.CMVnSpike, another replication-defective human adenovirus serotype 5-based vaccine vector encoding codon-optimized spike protein, to immunize C57BL/6 mice im. Splenocytes were harvested at 8 days after immunization and subjected to IFN-γ ELISPOT assay with pools of overlapping peptides from spike protein. Of all peptide pools screened, pools 9, 11, and 18 showed specific IFN-γ responses in C57BL/6. The remaining pools, as exemplified by pool 5, showed no specific response (Fig. 1A). Pools 9 and 11 were selected for mapping the individual peptide(s) responsible for stimulating IFN-γ expressing T cells. Peptide 87 (TSTGNYNYKYRYLRH, corresponding to S431–445) and peptide 88 (YNYKYRYLRHGKLRP, corresponding to S436–450) within pool 9 and peptide 105 (KNQCVNFNFNGLTGT, corresponding to S521–535) and peptide 106 (NFNFNGLTGTGVLTP, corresponding to S526–540) within pool 11 were identified as the major positive peptides responsible for specific stimulation of T cells to produce IFN-γ (Fig. 1B). Based on the SYFPEITHI algorithm (Rammensee et al., 1999), several potential CD8 T-cell epitopes for SARS-CoV spike protein in C57BL/6 mice were identified. Table 1 showed their sequences, positions, and scores. Indeed, YNYKYRYL (S436–443) completely present in peptides 87 and 88 was predicted to have strong binding affinity for H2-K^b; while VNFNFNGL (S525–532) completely present in peptide 105 and partially present in peptide 106 was predicted to have weak binding affinity for H2-K^b. Since ELISPOT assay cannot readily distinguish antigen-specific CD4 T-cell responses from antigen-specific CD8 T-cell responses, the immunized splenocytes were further subjected to intracellular IFN-γ staining after stimulating in vitro with peptides 87 and 105 (Fig. 2). Subsequently, the predicted optimal CD8 T-cell epitopes, YNYKYRYL resided in peptide 87 and VNFNFNGL resided in peptide 105, were synthesized and used to stimulate immunized splenocytes in vitro for IFN-γ secretion (Fig. 2). As predicted, YNYKYRYL and peptides 87 gave much stronger CD8 T-cell responses than VNFNFNGL and peptides 105. Furthermore, optimal epitopes, YNYKYRYL and VNFNFNGL, were able to induce IFN-γ responses in CD8 T cells either as effectively as or more effectively than their parental 15 mers, respectively. In conclusion, a single administration of a replication-defective human adenoviral vaccine vector encoding codon-optimized spike can generate strong spike-specific CD8 T-cell responses. We also identified two optimal CD8 T-cell epitopes of SARS-CoV spike in C57BL/6 mice.

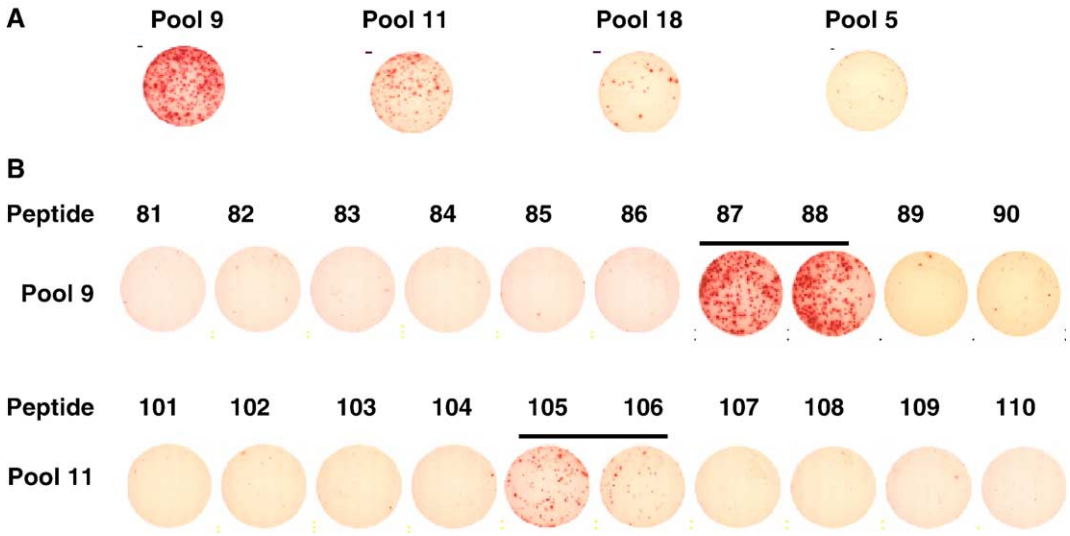

Fig. 1. IFN-γ ELISPOT assay to map T cell epitopes of SARS-CoV spike protein in C57BL6 mice. Mice were immunized with 5×10^{10} particles of AdHu5.CMVnSpike via im injection. 8 days after immunization, splenocytes were harvested and pooled from 5 mice and stimulated in vitro with either pools of overlapping peptides from spike protein (A) or individual peptides from positive pools (B) for IFN-γ ELISPOT assay. Experiments were done in duplicate and representative results were shown.

Determining the dose responses of two H-2^b-restricted CD8 T-cell epitopes for T cell activation

Immunized C57BL/6 splenocytes were in vitro stimulated with the two identified optimal CD8 T-cell epitopes at different concentrations ranging from 5 μg/ml to 5e−7 μg/ml, respectively. T cells activation was measured by intracellular IFN-γ staining (Fig. 3). It appeared that the minimal amounts of peptides needed to fully activate T cells in vitro were similar for both epitopes, even though the magnitude of T cell response with YNYKYRYL was much higher than that with VNFNFNGL.

Mapping of H-2^d-restricted CD8 T-cell epitopes in SARS-CoV spike protein

BALB/c mice were immunized in a similar fashion as C57BL/6 mice. Using ELISPOT assay, pools 7, 8, and 9 showed specific IFN-γ responses in BALB/c. The remaining pools, as exemplified by pool 5, showed no specific response (Fig. 4A). We focused on pools 8 and 9 because of the stronger responses observed with these pools. Subsequently, peptide 73 (FSTFKCYGVSATKLN, corresponding to S361–375) and peptide 74 (CYGVSATKLNDLCFS, corresponding to S366–380) within pool 8 and peptide 87 (TSTGNYNYKYRYLRH, corresponding to S431–445) and peptide 88 (YNYKYRYLRHGKLRP, corresponding to S436–450) within pool 9 were identified as the major positive peptides responsible for specific stimulation of T cells to produce IFN-γ (Fig. 4B). Based on the SYFPEITHI algorithm (Rammensee et al., 1999), several potential CD8 T-cell epitopes for SARS-CoV spike protein in BALB/c mice were identified. Table 2 showed their sequences, positions, and scores. Indeed, CYGVSATKL (S366–374) completely present in peptides 73 and 74 was predicted to have strong binding affinity for H2-K^d; while NYNYKYRYL (S435–443) completely present in peptide 87 and partially present in peptide 88 was predicted to have weak binding affinity for H2-K^d. The immunized splenocytes were also subjected to intracellular IFN-γ staining after stimulating in vitro with these positive 15 mers. Notably, there was no significant IFN-γ production from CD8 T cells stimulated with peptides 87 and 88 by intracellular IFN-γ staining (Fig. 5A), even though both peptides gave strong responses in IFN-γ ELISPOT assay (Fig. 4B). This suggested that peptides 87 and 88 may contain a CD4 T-

Table 1
Selected candidates of CD8 T-cell epitopes for SARS-CoV spike protein in C57BL/6

Peptide position	MHC class I	Peptide length	Peptide sequence	SYFPEITHI score
1156–1165	H-2D^b	10	A S V V N I Q K E I	30
695–704	H-2D^b	10	A I P T N F S I S I	27
779–787	H-2D^b	9	F G G F N F S Q I	27
436–443	H-2K^b	8	Y N Y K Y R Y L	29
884–891	H-2K^b	8	M A Y R F N G I	24
348–355	H-2K^b	8	C V A D Y S V L	22
525–532	H-2K^b	8	V N F N F N G L	21

The candidates of CD8 T-cell epitopes present in the positive 15 mers were shown in red.

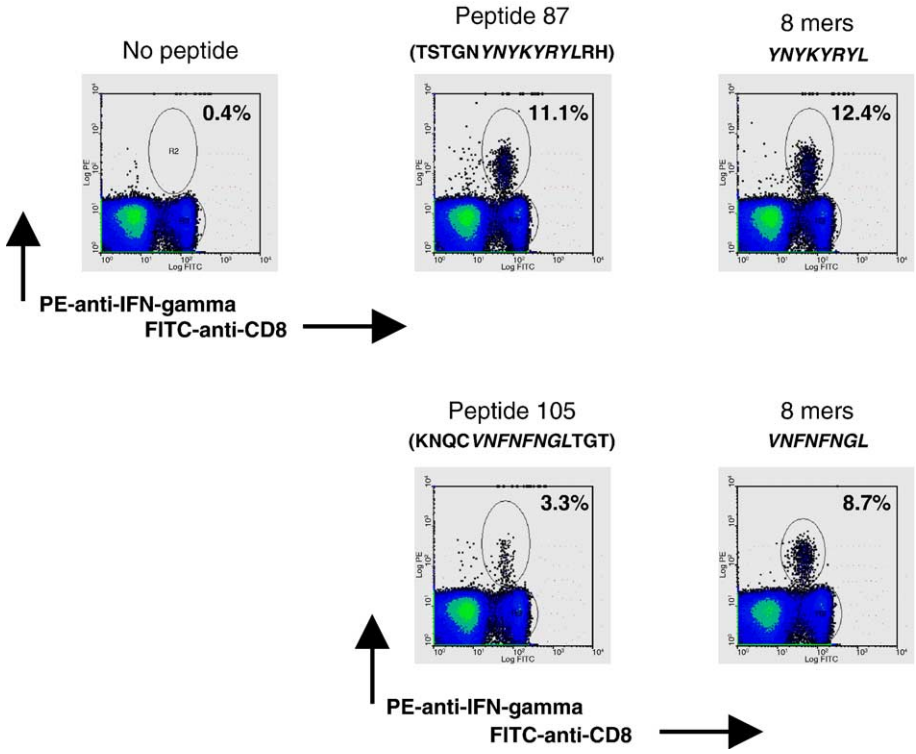

Fig. 2. Intracellular IFN-γ staining to confirm CD8 T-cell epitopes of SARS-CoV spike protein in C57BL/6 mice. Mice were immunized with 3×10^{10} particles of AdHu5.CMVnSpike via im injection. 10 days after immunization, splenocytes were harvested and pooled from 3 mice and stimulated with either 15 mers positive peptides or optimal 8 mers CD8 T-cell epitopes, as indicated, for 5 h. The immune response was evaluated by intracellular IFN-γ staining with PE-anti-IFN-γ and FITC-anti-CD8 antibodies. Numbers in the upper right corner of each graph represent the frequencies of IFN-γ-producing CD8 T cells. Experiments were done in duplicate and representative results were shown.

cell epitope that stimulated CD4 T-cell responses in IFN-γ ELISPOT assay. In contrast, peptide 74 can significantly stimulate CD8 T-cell responses (Fig. 5B). Subsequently, the predicted optimal CD8 T-cell epitope, CYGVSATKL resided in peptide 74, was synthesized and used to stimulate immunized splenocytes in vitro for IFN-γ secretion (Fig. 5B). The results indicated that peptide 74 indeed contained an H-2^d-restricted CD8 T-cell epitope. As expected, optimal epitope was able to induce IFN-γ responses in CD8 T cells more effectively than its parental 15 mer. Collectively, we have identified one optimal CD8 T-cell epitope of SARS-CoV spike in BALB/c mice. The dose responses of this

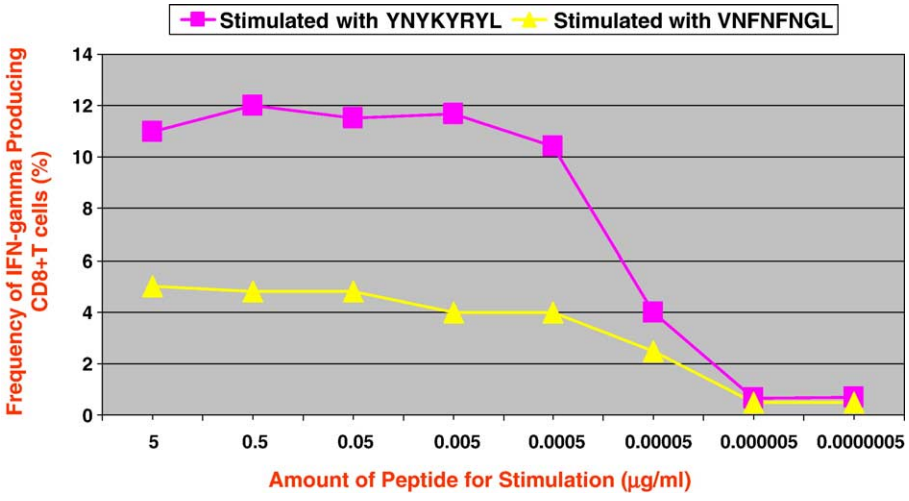

Fig. 3. Dose responses of H-2^b-restricted CD8 T-cell epitopes for T cell activation in vitro. C57BL/6 mice were immunized with 5×10^{10} particles of AdHu5.CMVnSpike via im injection. 8 days after immunization, splenocytes were harvested and pooled from 3 mice and stimulated with two identified CD8 T-cell epitopes at different concentrations, as indicated, respectively, for 5 h. T cell activation was evaluated by intracellular IFN-γ staining. Experiments were done in duplicate and representative results were shown.

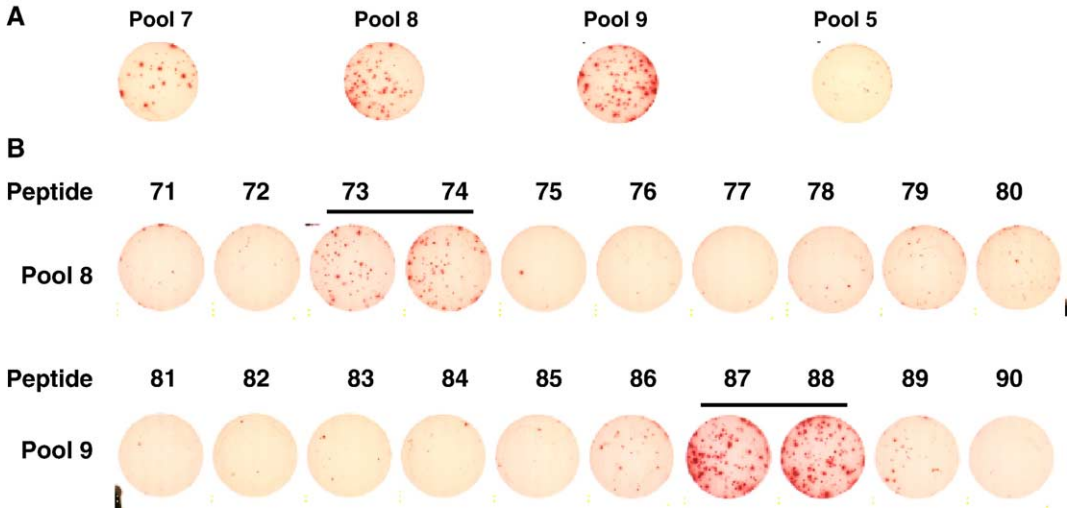

Fig. 4. IFN-γ ELISPOT assay to map T cell epitopes of SARS-CoV spike protein in BALB/c mice. Mice were immunized with 5×10^{10} particles of AdHu5.CMVnSpike via im injection. 8 days after immunization, splenocytes were harvested and pooled from 5 mice and stimulated in vitro with either pools of overlapping peptides from spike protein (A) or individual peptides from positive pools (B) for IFN-γ ELISPOT assay. Experiments were done in duplicate and representative results were shown.

CD8 T-cell epitope for T cells activation in vitro were also determined as described above (Fig. 6). The results suggested that about 100-fold more peptides were needed to fully activate T cells in vitro with this H-2^d-restricted CD8 T-cell epitope compared to those with two H-2^b-restricted CD8 T-cell epitopes (Fig. 3). Furthermore, peptides 87 and 88 may contain a CD4 T-cell epitope of SARS-CoV spike in BALB/c mice.

Identifying the potential CD4 T-cell epitope(s) of SARS-CoV spike protein in BALB/c

To confirm the presence of CD4 T-cell epitope(s) in peptides 87 and 88, the AdHu5.CMVnSpike-immunized splenocytes of BALB/c mice were harvested and in vitro stimulated with three 15 mers individually, including peptides 74, 87, and 88. The CD4 T-cell response was directly evaluated by intracellular cytokine staining with FITC-anti-CD4 and PE-anti-IFN-γ antibodies (Fig. 7). As expected, when stimulated with peptide 74, there was no detectable IFN-γ secretion from immunized CD4 T cells, even though a significant IFN-γ production from immunized non-CD4 T cells (CD8 T cells) was observed. More importantly, peptides 87 and 88 were able to stimulate immunized CD4 T cells to product IFN-γ. These results indicated that peptides 87 and 88 indeed contained a CD4 T-cell epitope of spike protein in BALB/c mice.

Increasing the immunogenicity of SARS-CoV spike protein in the context of replication-defective simian adenoviral vaccine carrier

To circumvent the potential problem that neutralizing antibodies to human adenovirus serotype 5 vector by previous natural infections will impair its efficacy as vaccine carrier, our lab recently developed a series of novel replication-defective adenoviral vaccine carriers based on simian serotypes (Fitzgerald et al., 2003; Pinto et al., 2003). AdC7, one of those carriers, is able to induce robust transgene-specific CD8 T-cell responses in immunized mice (Kobinger et al., submitted for publication). Three replication-defective simian adenovirus serotype 7-based vaccine vectors encoding either wild-type spike driven by CMV promoter (AdC7.CMVSpike) or codon-optimized spike driven by CMV promoter (AdC7.CMVnSpike) or codon-optimized spike driven by a hybrid promoter CAG2 (AdC7.CAG2nSpike) were created and used to immunize mice. CAG2 promoter was created by deleting a 955-bp

Table 2
Selected candidates of CD8 T-cell epitopes for SARS-CoV spike protein in BALB/c

Peptide position	MHC class I	Peptide length	Peptide sequence	SYFPEITHI score
366–374	H-2K^d	9	C Y G V S A T K L	29
162–170	H-2K^d	9	E Y I S D A F S L	27
480–489	H-2K^d	10	D Y G F Y T T T G I	24
597–606	H-2K^d	10	L Y Q D V N C T D V	24
435–443	H-2K^d	9	N Y N Y K Y R Y L	21
1071–1079	H-2L^d	9	F P R E G V F V F	24
308–316	H-2L^d	9	V P S G D V V R F	22

The candidates of CD8 T-cell epitopes present in the positive 15 mers were shown in red.

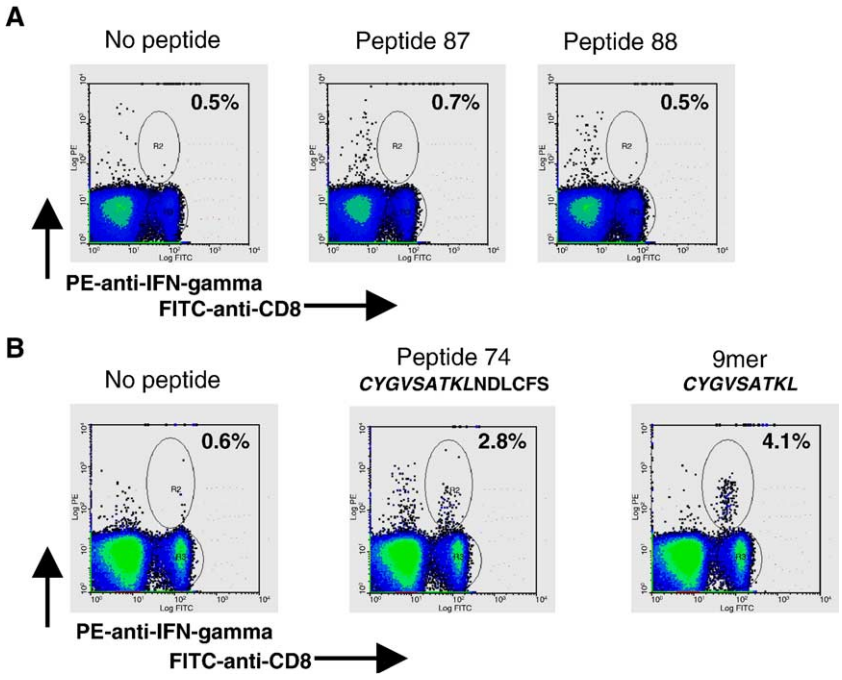

Fig. 5. Intracellular IFN-γ staining to confirm CD8 T-cell epitopes of SARS-CoV spike protein in BALB/c mice. Mice were immunized with 3×10^{10} particles of AdHu5.CMVnSpike via im injection. 10 days after immunization, splenocytes were harvested and pooled from 3 mice and stimulated with either 15 mers positive peptides or optimal 8 mers CD8 T-cell epitope, as indicated, for 5 h. The immune response was evaluated by intracellular IFN-γ staining with PE-anti-IFN-γ and FITC-anti-CD8 antibodies. Numbers in the upper right corner of each graph represent the frequencies of IFN-γ-producing CD8 T cells. Experiments were done in duplicate and representative results were shown.

*Apa*I/*Afl*II fragment from the original CAGGS promoter (Niwa et al., 1991). Similar to the negative results observed when AdHu5.CMVSpike was used to immunize mice, there was no detectable stimulation of an IFN-γ response in T cells with any of the identified positive pools using ELISPOT assay when AdC7.CMVSpike was used for immunization (data not shown). In contrast, spike-specific CD8 T-cell responses were observed when AdC7.CMVnSpike vector was used to immunize C57BL/6 mice (Fig. 8). However, the magnitude of spike-specific CD8 T-cell responses was much lower than that observed in mice immunized with AdHu5.CMVnSpike vector (Fig. 8). In order to further improve the immunogenicity of spike protein delivered by the simian adenoviral vaccine carrier, AdC7.CAG2nSpike vector was created. Chicken β-actin promoter has been shown to increase expression of SARS-CoV spike protein in transfected cells (Simmons et al., 2004). Spike-specific CD8 T-cell responses in C57BL/6 mice elicited by AdC7.CAG2nSpike vector were examined by intracellular IFN-γ staining (Fig. 8). The result indicated

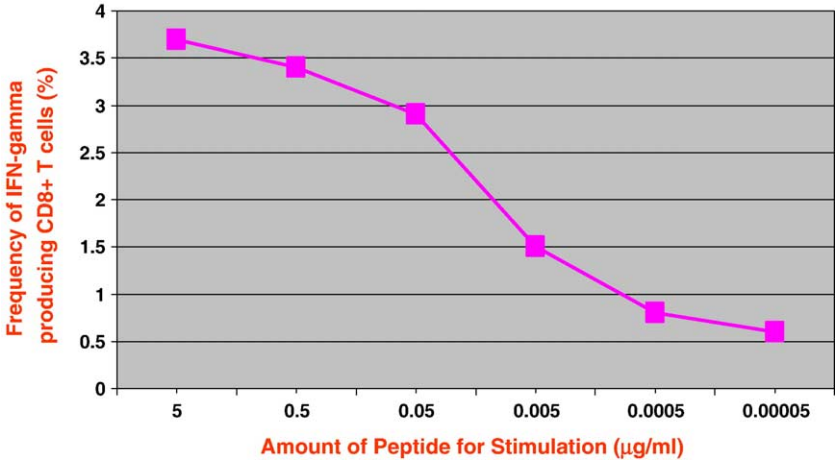

Fig. 6. Dose responses of H-2^d-restricted CD8 T-cell epitope for T cell activation in vitro. BALB/c mice were immunized with 5×10^{10} particles of AdHu5.CMVnSpike via im injection. 8 days after immunization, splenocytes were harvested and pooled from 3 mice and stimulated with the identified CD8 T-cell epitope at different concentrations, as indicated, for 5 h. T cell activation was evaluated by intracellular IFN-γ staining. Experiments were done in duplicate and representative results were shown.

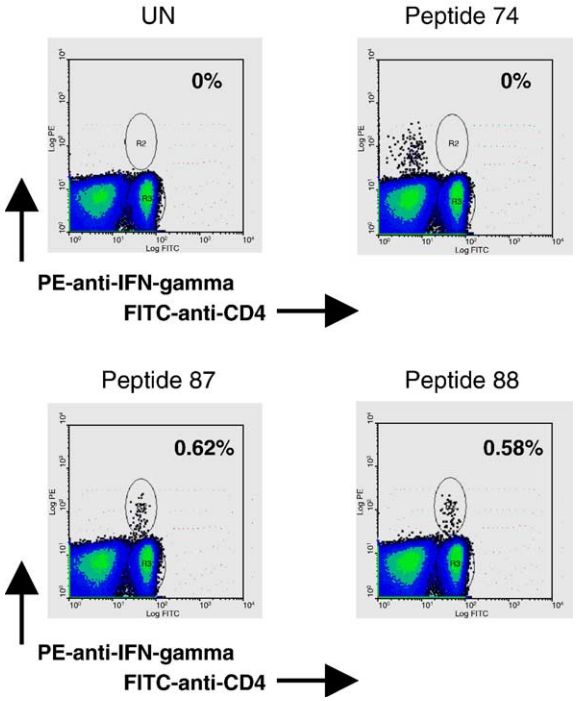

Fig. 7. Intracellular IFN-γ staining to confirm the presence of CD4 T-cell epitope of SARS-CoV spike protein in BALB/c mice. Mice were immunized with 5×10^{10} particles of AdHu5.CMVnSpike via im injection. 10 days after immunization, splenocytes were harvested and pooled from 3 mice and stimulated with 15 mers, as indicated, for 5 h. The immune response was evaluated by intracellular IFN-γ staining with PE-anti-IFN-γ and FITC-anti-CD4 antibodies. Numbers in the upper right corner of each graph represent the frequencies of IFN-γ-producing CD4 T cells. Experiments were done in duplicate and representative results were shown.

that codon and promoter optimizations can greatly enhance the overall immunogenicity of SARS-CoV spike protein in the context of simian adenoviral vaccine carrier. More importantly, a single administration of an optimized SARS-CoV spike vaccine vector based on a replication-defective simian adenovirus can generate strong spike-specific CD8 T-cell responses in mice.

Correlating the immunogenicity of SARS-CoV spike protein to its expression

It has been shown that increased expression of codon-optimized HIV gag protein is responsible for its enhanced immunogenicity in mice (Deml et al., 2001; Gao et al., 2003a). Therefore, it was of interest to determine whether optimization of transgene expression cassette, including codon and promoter optimizations, in the context of replication-deficient adenoviral vaccine vectors also resulted in the increased expression of spike protein in vitro. Expression of spike protein in Ad vector-infected 293T cells was examined by Western blot analysis and the bands representing spike protein were quantified by Image-Quant5.1. Forty-three-fold increase of protein expression was achieved when CAG2 promoter, instead of CMV promoter, was used in the simian adenoviral C7 vector (Fig. 9, lanes 4 and 6), while 7-fold increase of protein expression was achieved when codon optimization of spike protein was applied (Fig. 9, lanes 5 and 6). Overall, the results supported that the enhanced immunogenicity of spike in mice injected with AdHu5.CMVnSpike and AdC7.CAG2nSpike vaccine vectors (Fig. 8) indeed correlated to high expression of spike protein in cells infected with these vectors.

Discussion

SARS-CoV represents an emerging threat. Currently, no effective therapies or vaccines exist, and relatively little is known about the pathogenesis of the virus. It has been shown that 293T cells transfected with a functional receptor for SARS-CoV, angiotensin-converting enzyme 2 (ACE2), formed multinucleated syncytia with cells expressing spike protein (Li et al., 2003). More significantly, in the post-mortem lung tissue samples from patients who died from SARS, multinucleate giant cells of macrophage and epithelial origins have been observed within the damaged alveoli (Nicholls et al., 2003). In addition, CD8 T-cell responses are necessary for clearance of other CoVs, such as mouse hepatitis virus (Sussman et al., 1989; Williamson and Stohlman, 1990). Therefore, eradication of SARS-CoV may not be achieved by humoral response alone and T cell-mediated immunity may be also required to clear infection. To study more closely cellular immune responses in murine model, MHC I-restricted CD8 T-cell epitopes need to be identified. In this study, we have identified two octamers (S436–443 and S525–532) as H-2^b-restricted CD8 T-cell epitopes and one nonamer (S366–374) as an H-2^d-restricted CD8 T-cell epitope. In C57BL/6 mice, one epitope is more dominant than the other in immunized mice. The strength of these epitopes appeared to correlate with the binding affinity to H2-K^b predicted by SYFPEITHI algorithm. In the preliminary study, splenocytes were collected from C57BL/6 mice immunized with AdC7.CAG2nSpike vector and in vitro cultured in the presence of the dominant H-2^b-restricted CD8 T-cell epitope for a week. Subsequently, the cultured splenocytes were subjected to ELISPOT assay stimulated with MC57SV cells (H-$2D^b$, a cell line derived from B6 mice) previously infected with either AdHu5.Null or AdHu5.CMVnSpike vector. The initial results indicated that cultured splenocytes were able to induce higher level of IFN-γ response after stimulated with AdHu5.CMVnSpike vector-infected MC57SV cells than after stimulated with AdHu5.Null vector-infected MC57SV cells. These data suggested that T cells raised to the epitope were able to recognize cells expressing the spike protein and that the identified CD8 epitope is naturally processed and presented by cells. Currently, additional experiments are underway to address this issue more thoroughly.

Coronaviruses are common and worldwide pathogens that infect a variety of mammals and birds. These viruses have been classified into three groups. Although SARS-

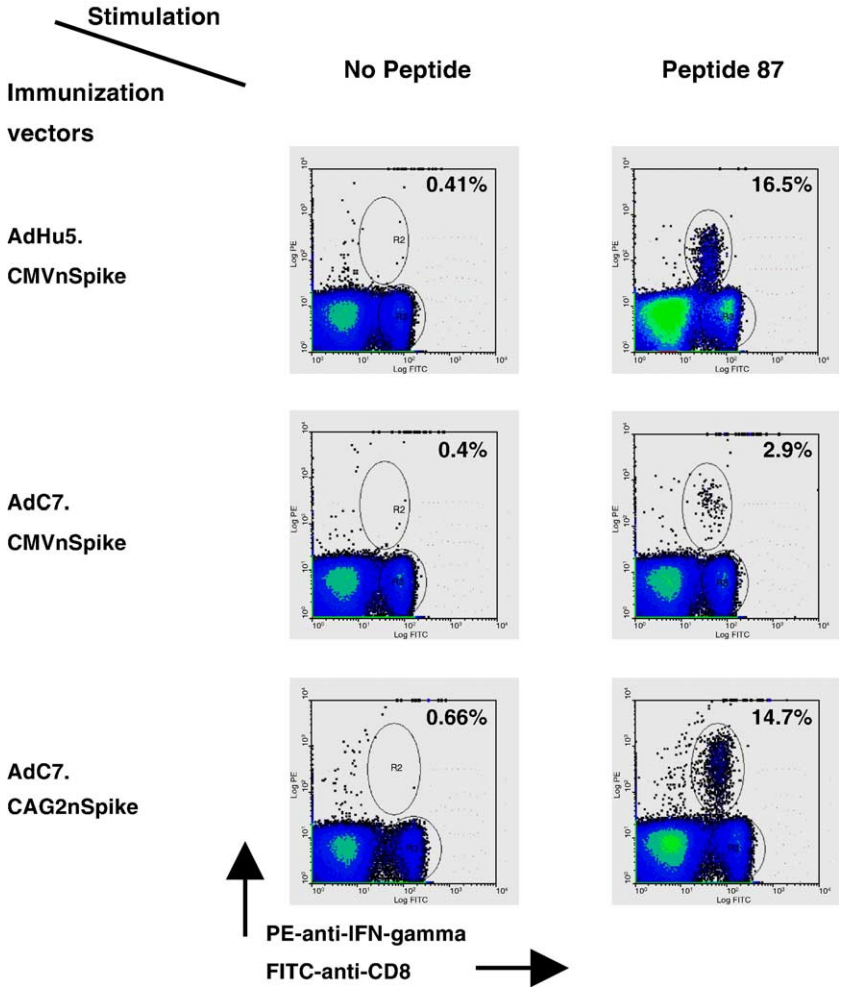

Fig. 8. Promoter optimization can further increase the immunogenicity of SARS-CoV spike protein delivered by the replication-defective simian adenoviral vaccine carrier. C57BL/6 mice were immunized with 5×10^{10} particles of vaccine vectors, including AdHu5.CMVnSpike, AdC7.CMVnSpike, and AdC7.CAG2nSpike, via im injection. 10 days after immunization, splenocytes were harvested and pooled from 3 mice in each group and stimulated in vitro with positive peptide for 5 h. The immune response was evaluated by intracellular IFN-γ staining with PE-anti-IFN-γ and FITC-anti-CD8 antibodies. Numbers in the upper right corner of each graph represent the frequencies of IFN-γ-producing CD8 T cells. Experiments were done in duplicate and representative results were shown.

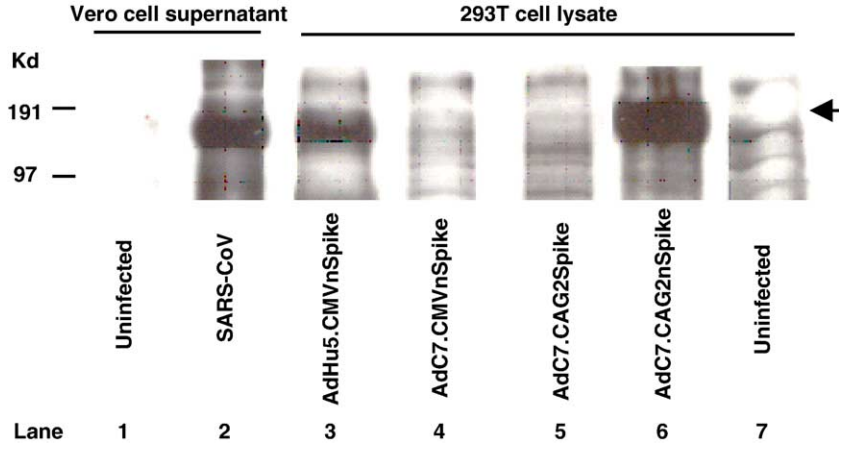

Fig. 9. Expression of SARS-CoV spike protein from a panel of replication-defective adenoviral vaccine vectors encoding spike in vitro. 293T cells were infected with each recombinant adenoviral vector encoding spike, as indicated. Infected cell lysates were made and separated by SDS–PAGE. Expression of spike protein was detected by Western blot using the whole serum collected from rabbits inoculated with purified spike protein. Uninfected cell lysate served as negative control and supernatant made from SARS-CoV-infected Vero cells served as positive control. Molecular weight markers were shown. The expected spike protein band was indicated by arrow.

CoV exhibits a similar genome structure, it is only distantly related to known CoVs and 'most like' Group 2 CoVs, which includes bovine, murine, and human viruses (Snijder et al., 2003). The most commonly studied coronavirus is mouse hepatitis virus (MHV), in part because the natural host for this infection, the mouse, is more easily managed in the laboratory. Two spike-specific CD8 T-cell epitopes (dominant S510–518 and subdominant S598–605) were identified in MHV-infected C57BL/6 mice (Bergmann et al., 1996; Castro and Perlman, 1995), while no spike-specific CD8 T-cell epitope has been recognized in MHV-infected BALB/c mice. The dominant S510–518 epitope is located in a hypervariable region of spike protein that appears to be readily deleted without loss of viability of MHV (Parker et al., 1989). In contrast, it was impossible to recover infectious virus with mutations in the subdominant S598–605 epitope (personal communication, M.M. Chua and S.R. Weiss). Examination of SARS-CoV spike sequence reveals that three CD8 T-cell epitopes are present in the S1 domain of the spike protein. Specifically, the dominant CD8 T-cell epitope in C57BL/6 (S436–443) and the CD8 T-cell epitope in BALB/c (S366–374) both reside in the minimal region of S1 required for interaction with its cellular receptor (Babcock et al., 2004; Wong et al., 2004). Several spike-specific CD4 T-cell epitopes have also been reported in MHV-infected mice (Heemskerk et al., 1995; Xue and Perlman, 1997). CD4 T cells are needed to help CD8 cells for viral clearance (Stohlman et al., 1998; Sussman et al., 1989; Williamson and Stohlman, 1990). In this study, we clearly showed that both peptide 87 and peptide 88 were able to specifically stimulate immunized CD4 T cells to produce IFN-γ in vitro. However, the exact sequences of CD4 T-cell epitope in the 431–450 region of spike protein remain to be further elucidated.

The first step in designing a genetic vaccine is to enhance the intrinsic immunogenicity of the selected target gene. In this study, we demonstrated that codon and promoter optimizations can greatly enhance the overall immunogenicity of spike protein in replication-defective human and simian adenoviral vaccine vectors. We showed that the expression of spike protein in vitro was greatly increased by expression cassette optimization. This provided useful information for developing spike-based SARS-CoV genetic vaccines. More importantly, this may provide us a platform to quickly generate genetic vaccine vectors based on simian adenovirus. Namely, CAG2 but not CMV promoter should be used in simian adenoviral vaccine vector. Currently, we are testing this hypothesis with other viral antigens. Since the CMV promoter has been widely used in human adenoviral vectors, it is surprising to see the inferior performance of CMV promoter in simian adenoviral vector. One possibility is that certain viral sequences only present in simian adenoviral vector have negative effects on CMV promoter activity. The other possibility is that certain inflammatory cytokines induced by simian adenoviral vector may inhibit CMV promoter activity.

Recently, a strategy involving priming with DNA vaccine and boosting with adenoviral vaccine vectors, each expressing a similar antigen, has resulted in the generation of unparalleled levels of specific immunity and afforded protection against infectious agents in animal models (Shiver et al., 2002; Sullivan et al., 2000). Nevertheless, DNA vaccines have performed poorly in clinical trials so far (MacGregor et al., 1998; Wang et al., 2001), and it is thus uncertain whether DNA vaccine prime followed by adenoviral vaccine vector boost will be as efficacious in humans as in preclinical experimental animal studies. Therefore, we developed an additional replication-defective adenoviral vaccine vector of chimpanzee origin to increase our repertoire of vaccine carriers that can be given sequentially (Fitzgerald et al., 2003; Pinto et al., 2003, 2004; Reyes-Sandoval et al., 2004; Roy et al., 2004; Xiang et al., 2002, 2003a, 2003b, 2003c). The toxicity of adenoviral vectors in vivo is directly related to the dose of injection. Therefore, a major benefit of prime/boost strategy is the potential to significantly reduce the dose for immunization in order to achieve a high level of immune responses. In this study, we showed that both AdHu5.CMVnSpike and AdC7.CAG2nSpike vaccine vectors could induce robust spike-specific CD8 T-cell responses in immunized mice alone. Currently, we are studying whether strong CD8 T-cell responses can be achieved when mice were primed and boosted with these heterologous adenoviral vaccine vectors at a much lower dose.

Materials and methods

Construction of molecular clones of replication-defective adenoviruses and production of vaccine vectors

Complementary DNA (cDNA) of spike gene for SARS-CoV was isolated by RT-PCR from the viral RNA of the SARS-CoV (Tor2 isolate). The PCR fragment was topo-cloned (Invitrogen, CA) and characterized by sequencing at SeqWright (SeqWright, TX), and was found to be 100% identical to the published sequence (Marra et al., 2003). Subsequently, Tor2 spike protein sequence was used as a template to design a synthetic spike gene sequence with human pattern of codon usage, according to Entelechon Backtranslation software tool. Finally, cloned Tor2 spike cDNA was used as a template and amplified with overlapping oligonucleotides in which human codon usage was introduced. Resulting overlapping PCR fragments were fused and full-length codon-optimized spike cDNA (nSpike) was created.

To generate the molecular clones of AdHu5 vectors, wild-type or codon-optimized spike insert was cloned into a pShuttle plasmid, followed by homologous recombination in bacterial cells using pAdEASY system (Invitrogen, CA). Molecular clones of all AdC7 vectors used in the study were created through a direct cloning and green-white selection

procedure as described elsewhere (Gao et al., 2003b; Roy et al., 2004). All of these molecular clones of replication-defective AdHu5 and AdC7 vectors were transfected into 293 cells for virus rescue. The rescued vectors were expanded to large-scale infections in 293 cells and purified by the standard CsCl gradient sedimentation method. Genome structures of the vectors were confirmed by restriction analysis. Infectivity of the vectors was determined by the standard plaque assay on 293 cells and levels of replication competent adenovirus (RCA) contaminants in the vector preparations were inspected as described previously (Gao et al., 2000).

Cells

293 and 293T cells were maintained in DMEM (Gibco-Life Technologies, Grand Island, NY) supplemented with antibiotic and 10% FBS (Hyclone, Logan, UT).

Mice

C57BL/6 and BALB/c mice (6–8 weeks old) were purchased from Charles River Laboratories (Wilmington, MA) and kept at the Animal Facility of The Wistar Institute (Philadelphia, PA).

Peptides

Peptide library derived from the SARS-CoV spike protein sequence was synthesized as 15 mers with 10-amino-acid overlap with the preceding peptide (Mimotopes, Victoria, Australia) and dissolved in DMSO at approximately 100 mg/ml. Pools of 10 consecutive peptides were made and stored at −20 °C. Peptides were used at the concentration of 5 μg/ml in all experiments except the dose response studies and DMSO concentrations were kept below 0.1% (v/v) in all final assay mixtures.

Immunization of mice

Groups of three to five mice were immunized with recombinant adenoviral vectors diluted in 100 μl phosphate-buffered saline (PBS) given im.

Expression of spike from infected 293T cells and Western blot analysis

293T cells were infected at 1000 particles/cell with each recombinant adenoviral vector encoding spike protein. Twenty-four hours later, cells were harvested and resuspended in lysis buffer and frozen at −20 °C. All samples were normalized to the lysate with the lowest total protein concentration by diluting with 4× SDS sample buffer (Invitrogen, Carlsbad, CA) plus 5% β-mercaptoethanol. The total protein content for all lysates was determined using the Bradford assay (BioRad, Melville, NY). Diluted samples were heated to 94 °C for 4 min and loaded onto an SDS–polyacrylamide gel. After electrophoresis, proteins were transferred onto PVDF membrane. Blots were blocked with 8% milk diluted in TBS/Tween 20 (TBS/T) for 45 min, washed three times (10 min each) with TBS/T, and probed with primary antibody at room temperature for 1 h. Primary antibody consisted of whole serum isolated from rabbits inoculated with purified spike protein diluted in 5% milk/TBS to a final concentration of 1:500. The blots were then washed three times with TBS/T before being incubated with secondary antibody for 45 min at room temperature and washed again three times with TBS/T. Secondary antibody was anti-rabbit HRP diluted in 5% milk/TBS to a final concentration of 1:2000 (Santa Cruz Biotechnology, Santa Cruz, CA). Protein Bands were developed using Super-Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and exposed on Kodak Biomax film. The Western blot image was scanned and the interested protein bands were quantified by ImageQuant5.1 (Molecular Dynamics).

IFN-γ ELISPOT assay

Assay was performed using ELISPOT Mouse Set (BD PharMingen, San Diego, CA) following the protocol provided by the vendor. Briefly, 96-well ELISPOT plate was coated with 5.0 μg/ml anti-mouse IFN-γ capture antibody overnight at 4 °C. Next day, wells were washed and blocked with complete culture medium for 2 h at room temperature. Splenocytes from immunized mouse were added to microwells along with spike-specific peptides. Cells were incubated at 37 °C and 5% CO_2 for 18–20 h. Control cells were incubated either without peptide or with nonspecific stimulator, SEB (200 ng/ml). Then wells were extensively washed with PBS containing 0.05% Tween 20 and subsequently incubated with 2.0 μg/ml biotinylated anti-mouse IFN-γ detection antibody for 2 h at room temperature. After washing, wells were incubated with 5 μg/ml Streptavidin-horseradish peroxidase antibody for 1 h at room temperature. Wells were washed again, and final substrate was added to wells. Color development was monitored and stopped by washing with water. After drying overnight at room temperature, wells were counted using an ELISPOT reader.

Intracellular IFN-γ staining

Splenocytes from immunized mice were stimulated with spike-specific peptides for 5 h at 37 °C and 10% CO_2 in the presence of 1 μl/ml Brefeldin A (GolgiPlug, BD PharMingen, San Diego, CA). Control cells were incubated without peptide. After washing, cells were stained with either a FITC-labeled anti-mouse CD8 antibody (BD PharMingen) or a FITC-labeled anti-mouse CD4 antibody (BD PharMingen). Then, cells were washed and permeabilized in Cytofix/Cytoperm (BD PharMingen) for 20 min on ice. Subse-

quently, cells were washed again and stained with a PE-labeled anti-mouse IFN-γ antibody (BD PharMingen). After extensively washing, cells were examined by two-color flow cytometry and data were analyzed by WinMDi software.

Acknowledgments

This work was supported by GlaxoSmithKline Pharmaceuticals. JMW is an inventor on patents licensed to various commercial entities.

References

Babcock, G.J., Esshaki, D.J., Thomas Jr., W.D., Ambrosino, D.M., 2004. Amino acids 270 to 510 of the severe acute respiratory syndrome coronavirus spike protein are required for interaction with receptor. J. Virol. 78 (9), 4552–4560.

Bergmann, C.C., Yao, Q., Lin, M., Stohlman, S.A., 1996. The JHM strain of mouse hepatitis virus induces a spike protein-specific Db-restricted cytotoxic T cell response. J. Gen. Virol. 77 (Pt. 2), 315–325.

Castro, R.F., Perlman, S., 1995. CD8+ T-cell epitopes within the surface glycoprotein of a neurotropic coronavirus and correlation with pathogenicity. J. Virol. 69 (12), 8127–8131.

De Groot, A.S., 2003. How the SARS vaccine effort can learn from HIV-speeding towards the future, learning from the past. Vaccine 21 (27–30), 4095–4104.

Deml, L., Bojak, A., Steck, S., Graf, M., Wild, J., Schirmbeck, R., Wolf, H., Wagner, R., 2001. Multiple effects of codon usage optimization on expression and immunogenicity of DNA candidate vaccines encoding the human immunodeficiency virus type 1 Gag protein. J. Virol. 75 (22), 10991–11001.

Fitzgerald, J.C., Gao, G.P., Reyes-Sandoval, A., Pavlakis, G.N., Xiang, Z.Q., Wlazlo, A.P., Giles-Davis, W., Wilson, J.M., Ertl, H.C., 2003. A simian replication-defective adenoviral recombinant vaccine to HIV-1 gag. J. Immunol. 170 (3), 1416–1422.

Fouchier, R.A., Kuiken, T., Schutten, M., van Amerongen, G., van Doornum, G.J., van den Hoogen, B.G., Peiris, M., Lim, W., Stohr, K., Osterhaus, A.D., 2003. Aetiology: Koch's postulates fulfilled for SARS virus. Nature 423 (6937), 240.

Gao, G.P., Engdahl, R.K., Wilson, J.M., 2000. A cell line for high-yield production of E1-deleted adenovirus vectors without the emergence of replication-competent virus. Hum. Gene Ther. 11 (1), 213–219.

Gao, F., Li, Y., Decker, J.M., Peyerl, F.W., Bibollet-Ruche, F., Rodenburg, C.M., Chen, Y., Shaw, D.R., Allen, S., Musonda, R., Shaw, G.M., Zajac, A.J., Letvin, N., Hahn, B.H., 2003a. Codon usage optimization of HIV type 1 subtype C gag, pol, env, and nef genes: in vitro expression and immune responses in DNA-vaccinated mice. AIDS Res. Hum. Retroviruses 19 (9), 817–823.

Gao, G., Zhou, X., Alvira, M.R., Tran, P., Marsh, J., Lynd, K., Xiao, W., Wilson, J.M., 2003b. High throughput creation of recombinant adenovirus vectors by direct cloning, green-white selection and I-Sce I-mediated rescue of circular adenovirus plasmids in 293 cells. Gene Ther. 10 (22), 1926–1930.

Heemskerk, M.H., Schoemaker, H.M., Spaan, W.J., Boog, C.J., 1995. Predominance of MHC class II-restricted CD4+ cytotoxic T cells against mouse hepatitis virus A59. Immunology 84 (4), 521–527.

Hofmann, H., Hattermann, K., Marzi, A., Gramberg, T., Geier, M., Krumbiegel, M., Kuate, S., Uberla, K., Niedrig, M., Pohlmann, S., 2004. S protein of severe acute respiratory syndrome-associated coronavirus mediates entry into hepatoma cell lines and is targeted by neutralizing antibodies in infected patients. J. Virol. 78 (12), 6134–6142.

Hogan, R.J., Gao, G.P., Rowe, T., Bell, P., Flieder, D., Paragas, J., Kobinger, G.P., Wivel, N.A., Crystal, R.G., Boyer, J.D., Feldmann, H., Voss, T.G., Wilson, J.M., 2004. Resolution of primary SARS-associated coronavirus infection requires Stat1. J. Virol. 78, 11416–11421.

Holmes, K.V., 2003. SARS coronavirus: a new challenge for prevention and therapy. J. Clin. Invest. 111 (11), 1605–1609.

Kobinger, G.P., Feldmann, E., Zhi, Y., Schumer, G., Gao, G., Feldmann, F., Jones, S., Wilson, J.M., submitted for publication. Protection mechanism of adenovirus vaccines in murine models of ebola infection and impact of pre-existing immunity to the vaccine carrier.

Ksiazek, T.G., Erdman, D., Goldsmith, C.S., Zaki, S.R., Peret, T., Emery, S., Tong, S., Urbani, C., Comer, J.A., Lim, W., Rollin, P.E., Dowell, S.F., Ling, A.E., Humphrey, C.D., Shieh, W.J., Guarner, J., Paddock, C.D., Rota, P., Fields, B., DeRisi, J., Yang, J.Y., Cox, N., Hughes, J.M., LeDuc, J.W., Bellini, W.J., Anderson, L.J., 2003. A novel coronavirus associated with severe acute respiratory syndrome. N. Engl. J. Med. 348 (20), 1953–1966.

Kuiken, T., Fouchier, R.A., Schutten, M., Rimmelzwaan, G.F., van Amerongen, G., van Riel, D., Laman, J.D., de Jong, T., van Doornum, G., Lim, W., Ling, A.E., Chan, P.K., Tam, J.S., Zambon, M.C., Gopal, R., Drosten, C., van der Werf, S., Escriou, N., Manuguerra, J.C., Stohr, K., Peiris, J.S., Osterhaus, A.D., 2003. Newly discovered coronavirus as the primary cause of severe acute respiratory syndrome. Lancet 362 (9380), 263–270.

Li, W., Moore, M.J., Vasilieva, N., Sui, J., Wong, S.K., Berne, M.A., Somasundaran, M., Sullivan, J.L., Luzuriaga, K., Greenough, T.C., Choe, H., Farzan, M., 2003. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. Nature 426 (6965), 450–454.

MacGregor, R.R., Boyer, J.D., Ugen, K.E., Lacy, K.E., Gluckman, S.J., Bagarazzi, M.L., Chattergoon, M.A., Baine, Y., Higgins, T.J., Ciccarelli, R.B., Coney, L.R., Ginsberg, R.S., Weiner, D.B., 1998. First human trial of a DNA-based vaccine for treatment of human immunodeficiency virus type 1 infection: safety and host response. J. Infect. Dis. 178 (1), 92–100.

Marra, M.A., Jones, S.J., Astell, C.R., Holt, R.A., Brooks-Wilson, A., Butterfield, Y.S., Khattra, J., Asano, J.K., Barber, S.A., Chan, S.Y., Cloutier, A., Coughlin, S.M., Freeman, D., Girn, N., Griffith, O.L., Leach, S.R., Mayo, M., McDonald, H., Montgomery, S.B., Pandoh, P.K., Petrescu, A.S., Robertson, A.G., Schein, J.E., Siddiqui, A., Smailus, D.E., Stott, J.M., Yang, G.S., Plummer, F., Andonov, A., Artsob, H., Bastien, N., Bernard, K., Booth, T.F., Bowness, D., Czub, M., Drebot, M., Fernando, L., Flick, R., Garbutt, M., Gray, M., Grolla, A., Jones, S., Feldmann, H., Meyers, A., Kabani, A., Li, Y., Normand, S., Stroher, U., Tipples, G.A., Tyler, S., Vogrig, R., Ward, D., Watson, B., Brunham, R.C., Krajden, M., Petric, M., Skowronski, D.M., Upton, C., Roper, R.L., 2003. The Genome sequence of the SARS-associated coronavirus. Science 300 (5624), 1399–1404.

Martina, B.E., Haagmans, B.L., Kuiken, T., Fouchier, R.A., Rimmelzwaan, G.F., Van Amerongen, G., Peiris, J.S., Lim, W., Osterhaus, A.D., 2003. Virology: SARS virus infection of cats and ferrets. Nature 425 (6961), 915.

Nicholls, J.M., Poon, L.L., Lee, K.C., Ng, W.F., Lai, S.T., Leung, C.Y., Chu, C.M., Hui, P.K., Mak, K.L., Lim, W., Yan, K.W., Chan, K.H., Tsang, N.C., Guan, Y., Yuen, K.Y., Peiris, J.S., 2003. Lung pathology of fatal severe acute respiratory syndrome. Lancet 361 (9371), 1773–1778.

Niwa, H., Yamamura, K., Miyazaki, J., 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 108 (2), 193–199.

Parker, S.E., Gallagher, T.M., Buchmeier, M.J., 1989. Sequence analysis reveals extensive polymorphism and evidence of deletions within the E2 glycoprotein gene of several strains of murine hepatitis virus. Virology 173 (2), 664–673.

Pinto, A.R., Fitzgerald, J.C., Giles-Davis, W., Gao, G.P., Wilson, J.M., Ertl, H.C., 2003. Induction of CD8+T cells to an HIV-1 antigen through a prime boost regimen with heterologous E1-deleted adenoviral vaccine carriers. J. Immunol. 171 (12), 6774–6779.

Pinto, A.R., Fitzgerald, J.C., Gao, G.P., Wilson, J.M., Ertl, H.C., 2004. Induction of CD8+T cells to an HIV-1 antigen upon oral immunization of mice with a simian E1-deleted adenoviral vector. Vaccine 22 (5–6), 697–703.

Rammensee, H., Bachmann, J., Emmerich, N.P., Bachor, O.A., Stevanovic, S., 1999. SYFPEITHI: database for MHC ligands and peptide motifs. Immunogenetics 50 (3–4), 213–219.

Reyes-Sandoval, A., Fitzgerald, J.C., Grant, R.L., Roy, S., Xiang, Z.Q., Li, Y., Gao, G.P., Wilson, J.M., Ertl, H.C.J., 2004. HIV-1 specific immune responses in primates upon sequential immunization with adenoviral vaccine carriers of human and simian serotypes. J. Virol. 78 (14), 7392–7399.

Rota, P.A., Oberste, M.S., Monroe, S.S., Nix, W.A., Campagnoli, R., Icenogle, J.P., Penaranda, S., Bankamp, B., Maher, K., Chen, M.H., Tong, S., Tamin, A., Lowe, L., Frace, M., DeRisi, J.L., Chen, Q., Wang, D., Erdman, D.D., Peret, T.C., Burns, C., Ksiazek, T.G., Rollin, P.E., Sanchez, A., Liffick, S., Holloway, B., Limor, J., McCaustland, K., Olsen-Rasmussen, M., Fouchier, R., Gunther, S., Osterhaus, A.D., Drosten, C., Pallansch, M.A., Anderson, L.J., Bellini, W.J., 2003. Characterization of a novel coronavirus associated with severe acute respiratory syndrome. Science 300 (5624), 1394–1399.

Roy, S., Gao, G.P., Lu, Y., Zhou, X., Lock, M., Calcedo, R., Wilson, J.M., 2004. Characterization of a family of chimpanzee adenoviruses and development of molecular clones for gene transfer vectors. Hum. Gene Ther. 15 (5), 519–530.

Shiver, J.W., Fu, T.M., Chen, L., Casimiro, D.R., Davies, M.E., Evans, R.K., Zhang, Z.Q., Simon, A.J., Trigona, W.L., Dubey, S.A., Huang, L., Harris, V.A., Long, R.S., Liang, X., Handt, L., Schleif, W.A., Zhu, L., Freed, D.C., Persaud, N.V., Guan, L., Punt, K.S., Tang, A., Chen, M., Wilson, K.A., Collins, K.B., Heidecker, G.J., Fernandez, V.R., Perry, H.C., Joyce, J.G., Grimm, K.M., Cook, J.C., Keller, P.M., Kresock, D.S., Mach, H., Troutman, R.D., Isopi, L.A., Williams, D.M., Xu, Z., Bohannon, K.E., Volkin, D.B., Montefiori, D.C., Miura, A., Krivulka, G.R., Lifton, M.A., Kuroda, M.J., Schmitz, J.E., Letvin, N.L., Caulfield, M.J., Bett, A.J., Youil, R., Kaslow, D.C., Emini, E.A., 2002. Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. Nature 415 (6869), 331–335.

Simmons, G., Reeves, J.D., Rennekamp, A.J., Amberg, S.M., Piefer, A.J., Bates, P., 2004. Characterization of severe acute respiratory syndrome-associated coronavirus (SARS-CoV) spike glycoprotein-mediated viral entry. Proc. Natl. Acad. Sci. U. S. A. 101 (12), 4240–4245.

Snijder, E.J., Bredenbeek, P.J., Dobbe, J.C., Thiel, V., Ziebuhr, J., Poon, L.L., Guan, Y., Rozanov, M., Spaan, W.J., Gorbalenya, A.E., 2003. Unique and conserved features of genome and proteome of SARS-coronavirus, an early split-off from the coronavirus group 2 lineage. J. Mol. Biol. 331 (5), 991–1004.

Stohlman, S.A., Bergmann, C.C., Lin, M.T., Cua, D.J., Hinton, D.R., 1998. CTL effector function within the central nervous system requires CD4+T cells. J. Immunol. 160 (6), 2896–2904.

Subbarao, K., McAuliffe, J., Vogel, L., Fahle, G., Fischer, S., Tatti, K., Packard, M., Shieh, W.J., Zaki, S., Murphy, B., 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 (7), 3572–3577.

Sullivan, N.J., Sanchez, A., Rollin, P.E., Yang, Z.Y., Nabel, G.J., 2000. Development of a preventive vaccine for Ebola virus infection in primates. Nature 408 (6812), 605–609.

Sussman, M.A., Shubin, R.A., Kyuwa, S., Stohlman, S.A., 1989. T-cell-mediated clearance of mouse hepatitis virus strain JHM from the central nervous system. J. Virol. 63 (7), 3051–3056.

van Ginkel, F.W., McGhee, J.R., Liu, C., Simecka, J.W., Yamamoto, M., Frizzell, R.A., Sorscher, E.J., Kiyono, H., Pascual, D.W., 1997. Adenoviral gene delivery elicits distinct pulmonary-associated T helper cell responses to the vector and to its transgene. J. Immunol. 159 (2), 685–693.

Wang, R., Epstein, J., Baraceros, F.M., Gorak, E.J., Charoenvit, Y., Carucci, D.J., Hedstrom, R.C., Rahardjo, N., Gay, T., Hobart, P., Stout, R., Jones, T.R., Richie, T.L., Parker, S.E., Doolan, D.L., Norman, J., Hoffman, S.L., 2001. Induction of CD4(+) T cell-dependent CD8(+) type 1 responses in humans by a malaria DNA vaccine. Proc. Natl. Acad. Sci. U. S. A. 98 (19), 10817–10822.

Wang, Y.D., Sin, W.Y., Xu, G.B., Yang, H.H., Wong, T.Y., Pang, X.W., He, X.Y., Zhang, H.G., Ng, J.N., Cheng, C.S., Yu, J., Meng, L., Yang, R.F., Lai, S.T., Guo, Z.H., Xie, Y., Chen, W.F., 2004. T-cell epitopes in severe acute respiratory syndrome (SARS) coronavirus spike protein elicit a specific T-cell immune response in patients who recover from SARS. J. Virol. 78 (11), 5612–5618.

Williamson, J.S., Stohlman, S.A., 1990. Effective clearance of mouse hepatitis virus from the central nervous system requires both CD4+ and CD8+ T cells. J. Virol. 64 (9), 4589–4592.

Wong, S.K., Li, W., Moore, M.J., Choe, H., Farzan, M., 2004. A 193-amino acid fragment of the SARS coronavirus S protein efficiently binds angiotensin-converting enzyme 2. J. Biol. Chem. 279 (5), 3197–3201.

Xiang, Z.Q., Yang, Y., Wilson, J.M., Ertl, H.C., 1996. A replication-defective human adenovirus recombinant serves as a highly efficacious vaccine carrier. Virology 219 (1), 220–227.

Xiang, Z., Gao, G., Reyes-Sandoval, A., Cohen, C.J., Li, Y., Bergelson, J.M., Wilson, J.M., Ertl, H.C., 2002. Novel, chimpanzee serotype 68-based adenoviral vaccine carrier for induction of antibodies to a transgene product. J. Virol. 76 (6), 2667–2675.

Xiang, Z., Li, Y., Gao, G., Wilson, J.M., Ertl, H.C., 2003a. Mucosally delivered E1-deleted adenoviral vaccine carriers induce transgene product-specific antibody responses in neonatal mice. J. Immunol. 171 (8), 4287–4293.

Xiang, Z.Q., Gao, G.P., Li, Y., Wilson, J.M., Ertl, H.C., 2003b. T helper cell-independent antibody responses to the transgene product of an e1-deleted adenoviral vaccine require NK1.1 T cells. Virology 305 (2), 397–405.

Xiang, Z.Q., Gao, G.P., Reyes-Sandoval, A., Li, Y., Wilson, J.M., Ertl, H.C., 2003c. Oral vaccination of mice with adenoviral vectors is not impaired by preexisting immunity to the vaccine carrier. J. Virol. 77 (20), 10780–10789.

Xue, S., Perlman, S., 1997. Antigen specificity of CD4 T cell response in the central nervous system of mice infected with mouse hepatitis virus. Virology 238 (1), 68–78.

Yang, Y., Haecker, S.E., Su, Q., Wilson, J.M., 1996. Immunology of gene therapy with adenoviral vectors in mouse skeletal muscle. Hum. Mol. Genet. 5 (11), 1703–1712.