

SARS coronavirus nucleocapsid immunodominant T-cell epitope cluster is common to both exogenous recombinant and endogenous DNA-encoded immunogens

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Abstract

Correspondence between the T-cell epitope responses of vaccine immunogens and those of pathogen antigens is critical to vaccine efficacy. In the present study, we analyzed the spectrum of immune responses of mice to three different forms of the SARS coronavirus nucleocapsid (N): (1) exogenous recombinant protein (N-GST) with Freund's adjuvant; (2) DNA encoding unmodified N as an endogenous cytoplasmic protein (pN); and (3) DNA encoding N as a LAMP-1 chimera targeted to the lysosomal MHC II compartment (p-LAMP-N). Lysosomal trafficking of the LAMP/N chimera in transfected cells was documented by both confocal and immunoelectron microscopy. The responses of the immunized mice differed markedly. The strongest T-cell IFN- γ and CTL responses were to the LAMP-N chimera followed by the pN immunogen. In contrast, N-GST elicited strong T cell IL-4 but minimal IFN- γ responses and a much greater antibody response. Despite these differences, however, the immunodominant T-cell ELISpot responses to each of the three immunogens were elicited by the same N peptides, with the greatest responses being generated by a cluster of five overlapping peptides, N_{76–114}, each of which contained nonameric H2^d binding domains with high binding scores for both class I and, except for N_{76–93}, class II alleles. These results demonstrate that processing and presentation of N, whether exogenously or endogenously derived, resulted in common immunodominant epitopes, supporting the usefulness of modified antigen delivery and trafficking forms and, in particular, LAMP chimeras as vaccine candidates. Nevertheless, the profiles of T-cell responses were distinctly different. The pronounced Th-2 and humoral response to N protein plus adjuvant are in contrast to the balanced IFN- γ and IL-4 responses and strong memory CTL responses to the LAMP-N chimera. © 2005 Elsevier Inc. All rights reserved.

Keywords: SARS coronavirus; Nucleocapsid protein; N DNA construct; LAMP-N DNA construct; Recombinant N; Rodent; T cells; Antigen peptide epitopes; ELISpot

Abbreviations: SARS, severe acute respiratory syndrome; SARS CoV, SARS-associated coronavirus; N, nucleocapsid protein; N-GST, N protein fused to glutathione *S*-transferase; N-His, N protein fused to histidine; LAMP1, lysosome associated membrane protein-1; MHC, MHC class II containing compartments; p-hLAMP-N, p43-human LAMP1 SARS CoV N construct; p-N, p43-SARS CoV N construct.

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Introduction

Vaccines designed to prevent viral infection must promote the adaptive expansion of T cells that are associated with B- and T-cell long-term memory of epitopes identical to or cross-reactive with those of the natural pathogen (Welsh et al., 2004; Crotty and Ahmed, 2004). The processing and presentation of antigen epitopes of vaccine immunogens must therefore mimic that of pathogens. The conventional understanding has been

that epitopes presented by MHC class I are derived from endogenous cellular proteins that have been cleaved by proteasomal and post-proteasomal peptidases (Villadangos et al., 1999; Yewdell and Bennink, 2001; Kloetzel 2004) and that the epitope loading of MHC class II molecules generally involves exogenous proteins that are taken into antigen-presenting cells (APCs) by specialized endocytic receptors and processed by proteases of the endosomal/lysosomal compartments (Moreno et al., 1991; Bryant and Ploegh, 2004).

It has been widely reported that various antigen-processing pathways may affect immune epitope formation either positively or negatively and may influence the repertoire of T-cell epitope-specific immune responses either quantitatively or qualitatively (Pamer and Cresswell, 1998; Kessler et al., 2002; Lautwein et al., 2004; Kloetzel, 2004). Such specificity in antigen processing would suggest that the form and delivery of an immunogen can be critical to the generation of biologically effective antigen epitopes and thus to vaccine efficacy. There is, however, abundant evidence for the presentation of exogenous antigens by MHC class I and of endogenous proteins by MHC class II through a variety of alternative antigen trafficking and processing mechanisms, including specialized processing pathways (Reimann and Schirmbeck, 1999; Norbury et al., 2001; Bonifaz et al., 2002; Imai et al., 2004), cross priming and presentation (Brode and Macary, 2004; Tewari et al., 2005), alternative endolysosomal pathways (Brooks et al., 1991; Lich et al., 2000; Chen and Jondal, 2004), vesicular translocation (Reis e Sousa and Germain, 1995; Ackerman and Cresswell, 2004), and phagocytic or autophagic mechanisms for antigen delivery to both the MHC class I and II compartments (Houde et al., 2003; Guermonprez et al., 2003; Nimmerjahn et al., 2003; Rocha and Tanchot, 2004; Paludan et al., 2005).

Nevertheless, little is known about the consequences of these alternative antigen trafficking and processing pathways for the production of pathogen-specific antigen epitopes. Thus, an examination of T-cell epitopes is a critical early step in the development of a new generation of vaccines, including DNA vaccines, chimeras with other protein sequences, or epitope-based antigens, all of which involve antigens as non-viral structures that may access processing pathways that differ from those followed by the native pathogens. These issues regarding the pathways used for antigen delivery and processing are particularly relevant to MHC class II epitopes. Presentation of antigen epitopes and activation of CD4 T cells by MHC class II molecules are critical steps in generating memory B cell and CD8⁺ T-cell phenotypes (Wang and Livingstone, 2003; Sun et al., 2004), and failure to elicit immune memory is one of the recognized problems of conventional genetic vaccines (Maecker et al., 1998). This defect may reflect an inadequate level of antigen expression or restricted access to the MHC II processing and presentation compartments by DNA-encoded endogenous antigens that lack a dedicated trafficking pathway (Martins et al., 1995; Maecker et al., 1998; Oehen et al., 2000; Rush et al., 2002).

In response to this problem, we have characterized DNA vaccine constructs synthesized in the form of chimeras that link

the antigen sequences to the trafficking/targeting signals of the lysosome-associated membrane protein-1 (LAMP-1) (Chen et al., 1985). LAMP molecules are known to traffic to lysosomes and to specialized multilaminar vesicular compartments of immature APCs, termed MIIC, where MHC II antigen processing and the formation of antigenic peptide–MHC II complexes occur (Kleijmeer et al., 1997; Geuze 1998; Murk et al., 2004). Studies of several antigens (including HIV-1 Env and Gag, HPV E7, cytomegalovirus pp65, carcinoembryonic antigen, dengue preM/E and yellow fever Env, and the catalytic subunit of the telomerase reverse transcriptase (hTERT)) expressed from viral vectors and naked DNA or RNA have shown that LAMP-targeted antigens generated greater antigen-specific lymphoproliferative activity, antibody titers, and cytotoxic T-lymphocyte activities than do DNA constructs encoding wild-type antigens (Rowell et al., 1995; Wu et al., 1995; Ruff et al., 1997; Nair et al., 1998; Raviprakash et al., 2001; Bonini et al., 2001; Su et al., 2002; Lu et al., 2003; Marques et al., 2003; Chikhlikar et al., 2004; Barros de Arruda et al., 2004; Anwar et al., 2005; Su et al., 2002). Most recently, in a clinical study, patients who received dendritic cells transfected with RNA encoding an hTERT/LAMP chimera developed higher frequencies of hTERT-specific T-cell responses than did subjects receiving dendritic cells transfected with the unmodified hTERT template (Su et al., 2005).

Despite this abundance of evidence of increased immune responses to LAMP chimeras of antigen proteins, the biological consequences of the enhanced immune responses in relation to protective immunity have not been defined, and there remains a critical question of the identity of T-cell epitopes of LAMP-targeted antigens as compared to those of natural pathogens. LAMP trafficking is not a normal mechanism for antigen access to the MHC class II compartment of antigen-presenting cells, and the epitope specificity of antigen processing and presentation *in vivo* in comparison to other antigen delivery systems remains to be demonstrated.

In the present study, we have used the severe acute respiratory syndrome coronavirus (SARS CoV) nucleocapsid (N) as a model antigen to address this issue of immune response and T-cell epitope presentation as a function of antigen formulation. The N protein of 422 amino acids is abundantly expressed and highly immunogenic (Lau et al., 2004; Huang et al., 2004a). The primary function of this structural protein in virus assembly is to bind to SARS CoV RNA during the formation of the ribonucleoprotein complex (Huang et al., 2004b). Previous studies have shown that both the N protein (Lin et al., 2003; Zhao et al., 2005) and DNA encoded N (Kim et al., 2004; Zhu et al., 2004; Yang et al., 2004) elicit antibody and cytotoxic T-cell responses in mice. In this analysis of the effect of immunogen formulation and delivery on immune responses *in vivo*, we have utilized three forms of N delivery: (1) recombinant N-GST protein combined with Freund's adjuvant as a classical exogenous antigen under conditions of profound activation of the innate immune response; (2) N encoded in a DNA plasmid as an unmodified endogenous cytoplasmic/nuclear antigen; and (3) a DNA construct with N inserted into the luminal domain of the entire

LAMP-1 molecule as an unnatural endogenous antigen targeted to the luminal compartment of endosomal/lysosomal vesicles (Guarnieri et al., 1993).

Results

Expression of N in cells transfected with p-N and p-LAMP-N DNA plasmids

Validation of N expression by p-N and p-LAMP-N DNA constructs was carried out by analyzing cells transfected in vitro. cDNA encoding the N protein was prepared by RT-PCR with N-specific primers and SARS RNA purified from a Singapore clinical isolate (GenBank accession no. AY307165). The corresponding 1269-bp N sequence was inserted into the p43 vector (Kessler et al., 1996) to form the wild-type p-N DNA expression vector and into the p43-hLAMP-1 construct in a position 5'-proximal to the LAMP transmembrane domain

to make the p-hLAMP-N vector (Fig. 1A). Western blot analysis of N in cell lysates and culture supernatants of transfected COS-7 cells showed the presence of the ~42 kDa N protein at comparable levels in cell lysates and culture supernatants of p-N and p-hLAMP-N-transfected COS-7 cells and of various forms of the higher molecular weight LAMP-N chimera in the cell lysate and culture supernatant of p-hLAMP-N-transfected cells (Fig. 1B). The N-like protein in the cell lysate of p-hLAMP-N-transfected cells is attributed to the cleavage of N from the protease-resistant LAMP at some stage in the cellular trafficking of the molecule. Intact or near-intact HIV-1 Gag protein was also observed with cells transfected with the corresponding LAMP/Gag DNA vaccine construct (Marques et al., 2003). Several forms of the larger LAMP-N molecule were also present in both the cell lysate and culture supernatants of the transfected cells. The intact LAMP-N is found at about 150 kDa, and the other bands are attributed to various degradation products. However, we are cautious about

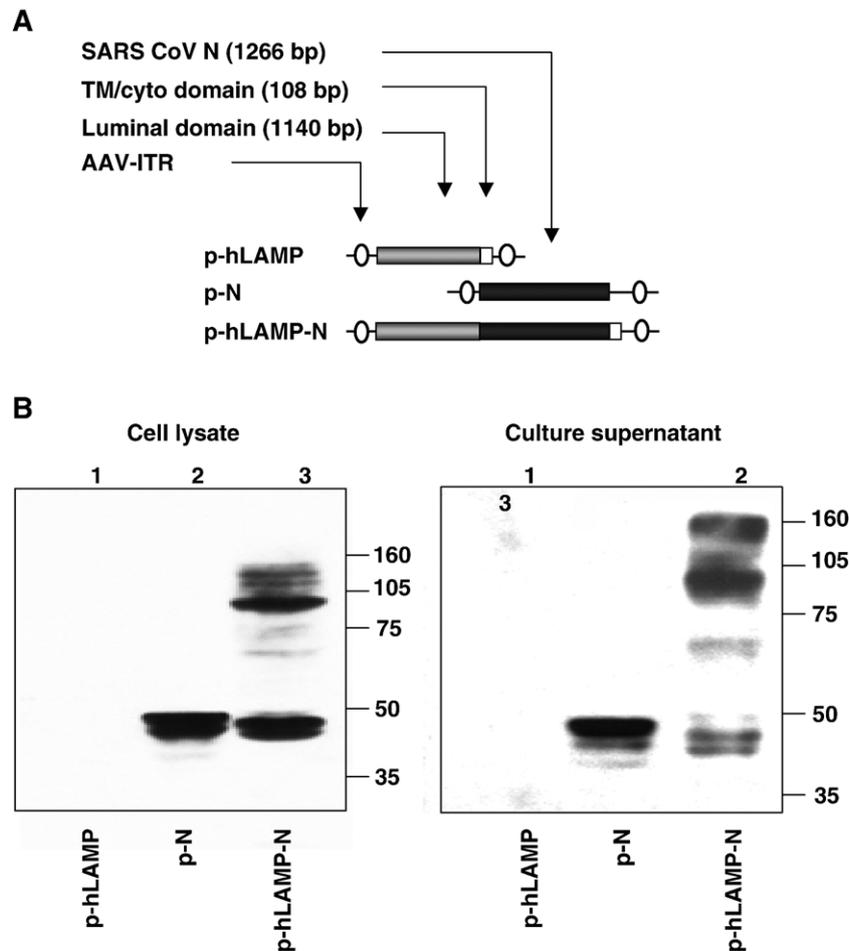


Fig. 1. Construction and expression of DNA plasmids encoding SARS CoV N and hLAMP-N. Transgenic N and hLAMP-N chimera are expressed at comparable levels in cell lysate and culture supernatant of transfected cells. (A) Schematic representation of the plasmid constructs. Arrows indicate the schematic position of the various sequences: SARS CoV native nucleocapsid (SARS CoV N), hLAMP1 luminal domain (luminal domain), the hLAMP1 transmembrane and cytoplasmic domains (TM/cyto), and the adeno-associated virus inverted terminal repeat sequences (AAV ITR). p-hLAMP: a control plasmid encoding the full human LAMP1 luminal, transmembrane (TM) and cytoplasmic (cyto) domains in the p43 expression vector. p-N: the unmodified SARS CoV N sequence in the p43 plasmid. p-hLAMP-N: SARS CoV N sequences inserted into the full hLAMP1 sequence proximal to the hLAMP TM domain. Boxed areas represent the open reading frames as indicated. The ovals indicate the AAV-ITR sequences of the p43 plasmid. (B) N and hLAMP-N protein expression in transfected monkey kidney COS-7 cells. Western blot analysis of p-hLAMP (control), p-N and p-hLAMP-N-transfected cells with anti-N-GST polyclonal serum showed comparable amounts of N and hLAMP-N protein in both the cell lysate and culture supernatant. The positions of the protein markers in kDa are shown on the right.

drawing conclusions from the *in vitro* experiments as such experiments may have little relationship to the expression of the immunogen in the cells transfected *in vivo*.

Confocal and immune electron microscopy

The presumed cell trafficking pathway for the newly synthesized N, which lacks an endoplasmic reticulum translocation (signal) sequence, is to the cell cytoplasm. In contrast, the LAMP-N chimera is expected to traffic to endosomal/lysosomal vesicles because of the LAMP vesicular trafficking sequences. We used confocal microscopy to validate these trafficking destinations for the immunoreactive unmodified N antigens and N of the LAMP-N chimera expressed in transfected mouse LB 27.4 B cells and human melanoma Mel-Juso cells, comparing their cellular distribution to that of the endogenously expressed LAMP-1 and MHC class II proteins (Fig. 2). The colocalization of endogenous LAMP and MHC class II proteins in vesicular compartments of APCs has been extensively documented (Kleijmeer et al., 1996; Geuze, 1998; Murk et al., 2004). Trafficking of the LAMP-N chimera protein to the LAMP and MHC II compartments of the transfected cells was validated by the colocalization of the LAMP-N chimera (labeled by anti-N antibody) with endogenous LAMP-1 and MHC class II in both mouse B (Figs. 2A–F) and human melanoma (Figs. 2M–O) cells. Endogenous MHC class II of the B cells was distributed both at the periphery of the cell and in intracellular foci, in accordance with the known maturation trafficking of vesicular MHC class II molecules to the cell surface. Colocalization of LAMP-N with MHC class II of B cells was restricted to the intracellular foci in the endosomal–lysosomal compartment and not with the mature MHC class II at the plasma membrane (Figs. 2D–

F). MHC class II and LAMP-N in transfected melanoma cells were both densely present throughout the cytoplasm and were markedly colocalized (Figs. 2M–O). Unmodified N was densely expressed throughout the cytoplasm of the transfected cells, again in apparently vesicular structures, but with little or no colocalization with either the endogenous LAMP-1 (Figs. 2G–I) or MHC class II (Figs. 2J–L) in B cells or melanoma cells (Figs. 2P–R).

Greater definition of the localization of N in transfected cells was obtained by analyzing of immunolabeled ultrathin cryosections of mouse LB 27.4 B cells. The endocytic compartments of these B cells are morphologically similar to those of other reported mouse B-cell lines (6H5.DM and A20.A^b), with well-defined multivesicular and multilaminar MIICs and end organelles (Geuze, 1998). Double immunogold labeling of the transfected B cells confirmed that N of the LAMP-N chimera was present with both endogenous LAMP and MHC class II molecules in the MIIC multilaminar vesicles (Figs. 3A, B). In contrast, unmodified N was predominantly localized in the cytoplasm or in vacuolated structures with no evident colocalization with LAMP or MHC class II (Figs. 3C–E). Remarkably, lower power magnification showed that some of the cytoplasmic vesicles contained large amounts of N. These vesicles may correspond to the strongly labeled vesicular structures shown by immunofluorescent microscopy, and the presence of N in these structures suggests a possible route for the secretion of the protein (Fig. 3E).

Mouse primary immune responses

N antigen in three forms, representing differences in antigen delivery, adjuvants, and peptide processing pathways, were administered according to the following protocols: (1) N as an

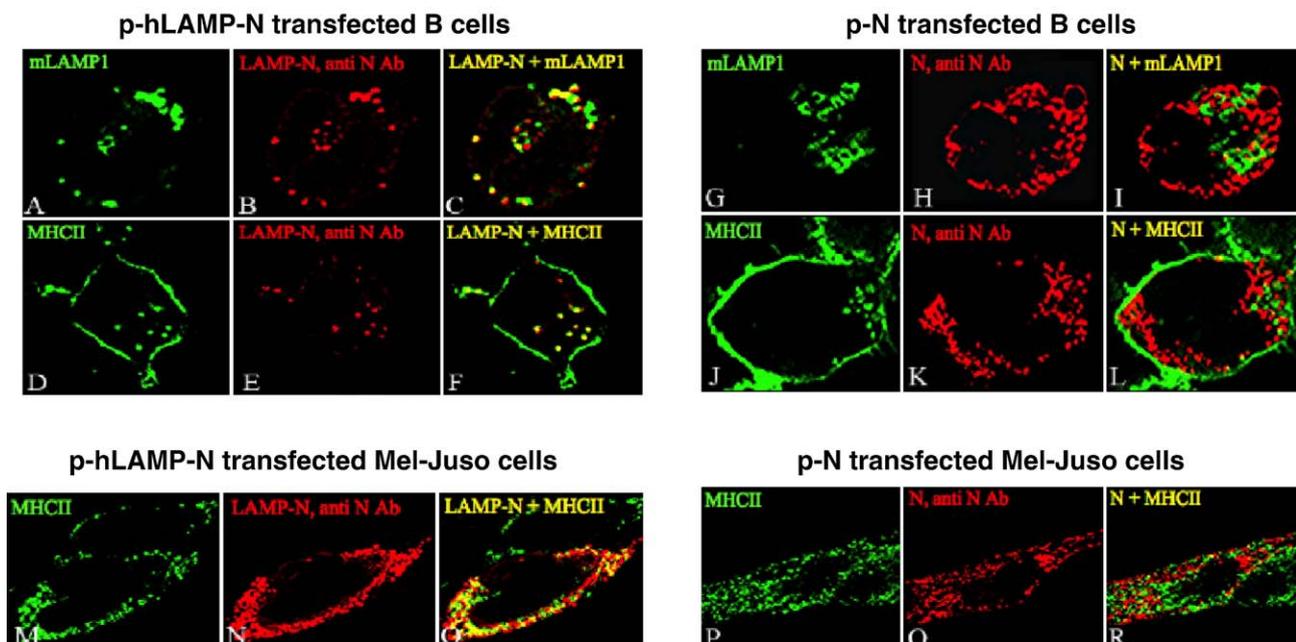


Fig. 2. Colocalization (yellow) of confocal images of immunolabeled transgenic N (red) with LAMP1 (green) and MHC II (green) in p-hLAMP-N-transfected murine B cells (A–F) and with MHC II in human Mel-Juso cells (M–O). Colocalization rarely seen in p-N-transfected cells (G–L, P–R).

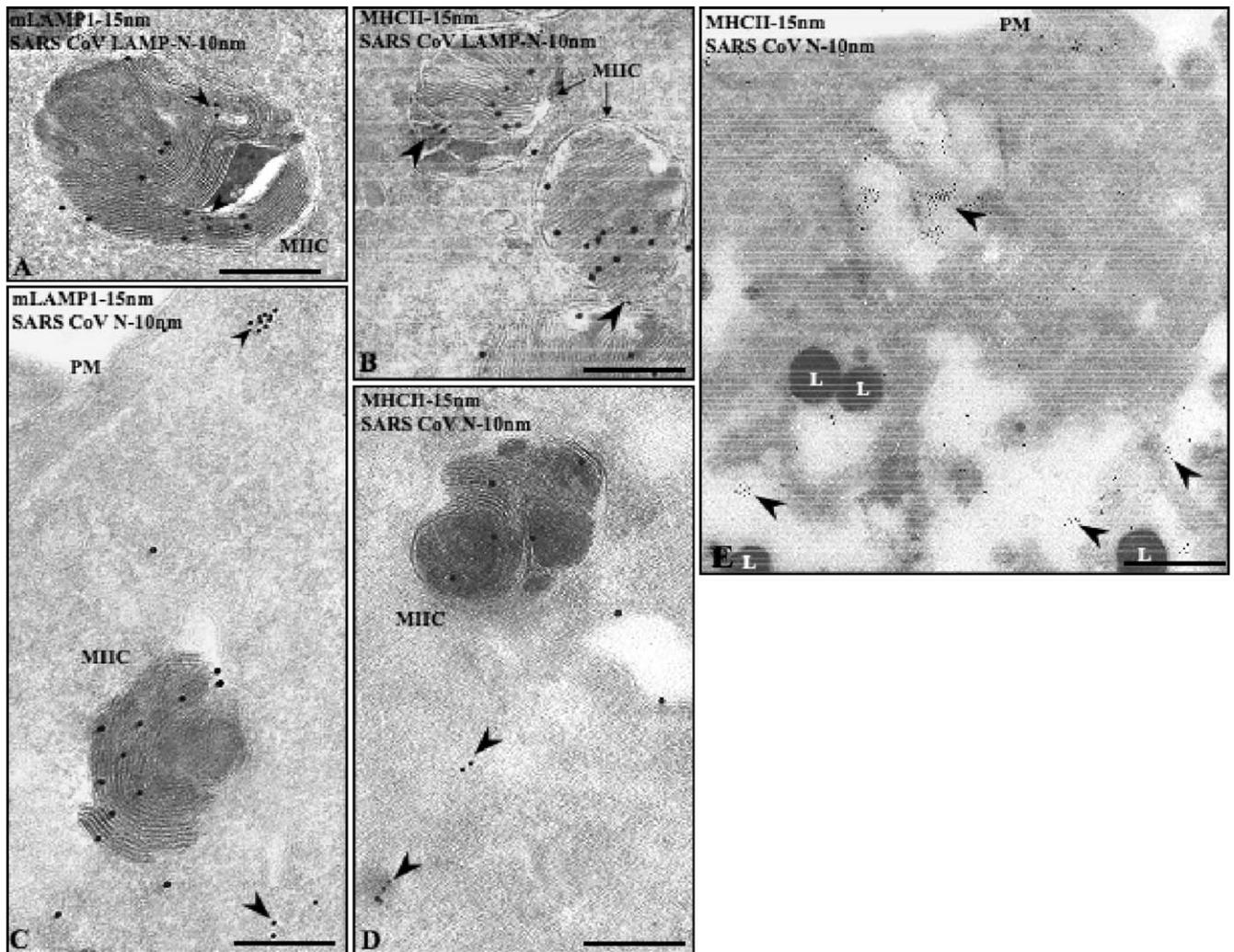


Fig. 3. Immunoelectron microscopy of murine B cells transfected with p-LAMP-N and p-N. N of p-hLAMP-N-transfected cells localized in typical MIIC multilaminar vesicles with endogenous LAMP (A) and MHC II (B). N of p-N-transfected cells not found with endogenous LAMP1 (C) or MHC II (D) but present as aggregates in cytoplasmic vesicles devoid of LAMP1 and MHC class II, sometimes in large numbers (E, low power). MIIC, MHC class II containing compartment; PM, plasma membrane; L, electron dense lysosomes. Scale bar was adjusted to 200 nm.

exogenous N-GST protein was administered three times, on day one and 3 and 6 weeks later, initially with CFA and then with IFA. (2) N as an unmodified endogenous, DNA-encoded, cytoplasmic protein, and (3) N as an artificial chimera encoded in the luminal domain of LAMP and targeted to cellular lysosomal and MHC II compartments were administered five times, on day 1, and 3, 6, 9, and 14 weeks later, without added adjuvant but subject to the molecular adjuvant effects of the DNA CpG motifs and the targeting of the LAMP-N chimera to the MHC II compartment. DNA encoding p-hLAMP was used as the negative control.

T-cell responses were assayed by IFN- γ ELISpot and ELISA assays using spleen cells collected 8 days after the final DNA immunization. For these assays, the T cells were stimulated with N-His protein as a positive control or with a library of overlapping N peptides, 15 to 20 amino acids in length. The results were confirmed in three experiments and in multiple assays with three independent sets of peptides from different sources. The IFN- γ responses were also assayed with an ELISA protocol, with similar results (data not shown). The

strongest IFN- γ responders were cells from mice immunized with p-hLAMP-N, with comparable but weaker responses by the p-N-immunized mice, and minimal responses by cells of the N-GST-immunized mice (Table 1; Fig. 4). The major grouping of IFN- γ responses was elicited by peptides of amino acid sequence N_{76–114}, encompassing 5 of the overlapping peptides. More recently, these results have also been confirmed in a new experimental protocol currently being conducted that also show that all of the peptides that encompass N_{76–114} elicit both CD4- and CD8-specific ELISpot responses (data not shown). Other dominant responses were to peptides N_{130–149} and N_{241–258} and the overlapping peptides N_{352–366} and N_{353–370}. All significant epitope-specific responses of the pN or N-GST-immunized mice were shared by the p-hLAMP-N-immunized mice. The weak IFN- γ responses of the N-GST protein immunized mice were observed only with peptides that elicited the strongest responses with splenocytes of the DNA-plasmid-immunized mice (Figs. 4A–C). This comparatively weak IFN- γ response of N-GST-immunized mice was in marked contrast to the strong IL-4 responses as shown below and to the

Table 1
T-cell IFN- γ ELISpot positive N-peptide sequences

N peptides			IFN- γ SFC/10 ⁶ splenocytes (mean \pm SD)		
Number	N-aa	Sequence	p-hLAMP-N	p-N	N-GST
11	76–93	NTNSGPDDQIGYYRRATR	57 \pm 2	3 \pm 2	6 \pm 1
12	80–99	GPDDQIGYYRRATRRVRGGD	429 \pm 8	266 \pm 18	46 \pm 12
13	84–101	QIGYYRRATRRVRGGDGK	377 \pm 11	209 \pm 6	55 \pm 6
14	92–109	TRRVRGGDGKMKELSPRW	309 \pm 13	212 \pm 7	24 \pm 5
15	100–114	GKMKELSPRWYFYLL	75 \pm 12	55 \pm 7	9 \pm 2
21	130–149	GIVWVATEGALNTPKDHIGT	106 \pm 4	61 \pm 8	5 \pm 3
35	241–258	QQQGQTVTKKSAAEASKK	131 \pm 20	23 \pm 3	2 \pm 1
51	352–366	ILLNKHIDAYKTFPP	137 \pm 10	34 \pm 8	38 \pm 4
52	353–370	LLNKHIDAYKTFPPTPEK	118 \pm 11	50 \pm 8	46 \pm 11

All ELISpot responses were to a group of 9 dominant peptides, shown by peptide number, amino acid region in N, sequence, and number of IFN- γ -positive cells of mice immunized with the three immunogens. A cluster (N_{76–114}) of 5 peptides of 15 to 20 aa, overlapping by 10 to 16, contains 3 of the strongest dominant epitopes.

exceptional strong antibody responses. The N-specific antibody titers to N-GST immunization on day 42 (after the first boost) was 1:25,000 and at day 63 (after the second boost) was 1:67,500, as compared to titers increasing to a maximum of 1:3200 on days 84 and 105 in response to immunization with the p-N and p-hLAMP-N DNA constructs.

Memory immune responses

Memory immune responses (total IgG antibody titers, CTL activity, and ELISpot IFN- γ and IL-4 responses) were examined by use of a separate experimental protocol with groups of mice treated with the same three N antigen preparations but receiving only three inoculations over 6 weeks. After an interval of 36 weeks, the memory immune response was activated by a single injection at week 42 of the p-N construct as a surrogate of virus infection. Antibody and T-cell ELISpot and CTL responses were measured immediately before p-N injection at 42 weeks and three times at week intervals thereafter (Figs. 5 and 6). The T-cell assays were conducted with the selected peptides that had elicited immunodominant responses in pilot experiments for IFN- γ , N_{80–99} and N_{241–258}; IL-4, N_{80–99} and N_{130–149}; and CTL, N_{80–99} and N_{353–370}. The residual T-cell IFN- γ and IL-4 responses of mice sacrificed on day 1 before p-N memory activation were very low and were significant only in mice initially immunized with p-hLAMP-N (Fig. 5). Following the recall p-N injection, increased IFN- γ and IL-4 responses were observed on day 8, reaching an apparent maximum on days 15 to 22. The strongest memory T-cell responses in all assays were those of mice initially immunized with p-hLAMP-N, with strong IFN- γ and IL-4 responses to both peptides. N-GST plus Freund's adjuvant elicited strong IL-4 responses but minimal IFN- γ responses, as seen for the primary response. The responses of mice initially immunized with p-N were uniformly lower than those seen for the LAMP chimera and the IL-4 responses to N-GST. However, the IFN- γ responses were greater for p-N than for N-GST. Memory CTL responses were strongest in p-hLAMP-N-immunized mice, with a much weaker response to p-N DNA immunization and little if any after immunization with N-GST with adjuvant (Fig. 6). Memory antibody responses followed the pattern of the primary response, with a much stronger IgG

titer of about 1:25,000 on days 15 and 22 following activation of the N-GST-immunized mice as compared to the responses of about 1:7000 by the p-hLAMP-N-immunized mice. There was relatively little response (1:1000) by mice initially immunized with p-N.

Correlation of ELISpot-positive peptides and computational predictions of N-specific H2^d binding motifs

Peptide epitope activation of T cells relies on receptor (TCR) recognition of complexes of antigen peptide sequences bound to the nine amino acids of the binding clefts of MHC class I and II molecules (Benacerraf, 1978). It is postulated that the kinetic stability of the MHC-peptide complexes is a key parameter that dictates immunodominance (Lazarski et al., 2005). In the further analysis of the T-cell epitope specificity of N, the predicted H2^d class I (H2-K^d, -L^d, -D^d) and class II (I-E^d, I-A^d) binding motifs of the 60 SARS N overlapping peptides were examined by a murine immunoinformatics system based on quantitative matrices, PRED^{BALB/c} (Zhang et al., 2005). Each of the 414 nine-amino-acid sequences of N was analyzed for binding to the core sequence of the 5 H2^d alleles. All of the nine IFN- γ ELISpot-positive peptides scored for a high probability of binding to class I and/or class II alleles (Table 2), and, in ongoing experiments, all of a new set of peptides encompassing N_{76–114} elicit both CD4- and CD8-specific responses (data not shown).

Discussion

In the present study, we have demonstrated that the dominant T-cell immune responses of mice immunized with the SARS CoV N protein plus adjuvant or with DNA encoding two different cellular trafficking forms of N are directed to the same peptide epitopes of all three immunogens. This finding points to the existence of versatile antigen-processing mechanisms that are not defined by the initial site of delivery of the protein, whether exogenous or endogenous, or the state of the innate responses to the immunogens. The data are consistent with multiple in vivo routes for antigen access to proteolytic processing compartments that produce the same or comparable MHC I and II peptide epitopes. This versatility would not

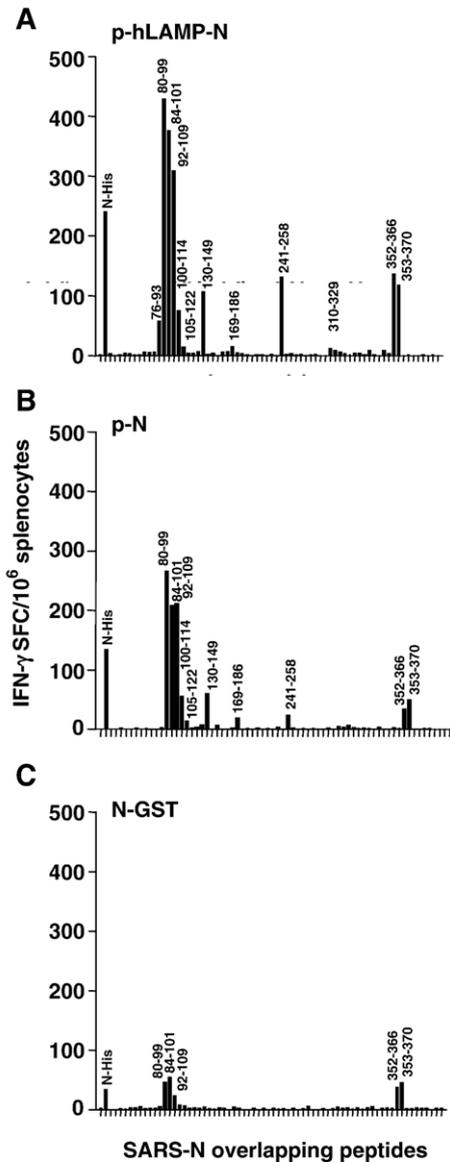


Fig. 4. Primary T-cell IFN- γ ELISpot responses of (A) p-hLAMP-N, (B) p-N, and (C) N-GST-immunized mice. As described in Materials and methods, mice were immunized five times with the DNA immunogens and three times with N-GST plus adjuvant, and the mice were sacrificed for ELISpot assay of antigen-specific splenocyte IFN- γ secretion 8 days after the final immunization. The splenocytes were stimulated with recombinant N-His protein and with N peptides from panels of overlapping peptides spanning the entire molecule, with mice immunized by hLAMP plasmid as the negative control. All significant T-cell peptide-specific IFN- γ responses elicited by each immunogen were stimulated by the same peptides, with the strongest responses to p-hLAMP-N and the weakest to N-GST.

preclude quantitative variation in epitope presentation as a result of differences in the levels of antigen, the degree of processing, or access to cellular compartments for antigen presentation. For example, in this study, the greater response to the LAMP-N chimera could be related to the quantitatively greater delivery of N to the MHC class II compartment of APCs. There can also be major differences in the repertoire or functionality of T cells responding to any given epitope, such as the differences in the IFN- γ and IL-4 responses of mice immunized with DNA or with N-GST

plus CFA. Others have reported similar findings that the same CTL-defined epitope peptide is generated by an exogenous protein and by plasmid DNA-encoded antigens (Schirmbeck et al., 1998) and that the efficiency of MHC class I cross-presentation of peptides derived from exogenous antigens is comparable to that for presentation by the classical MHC class II pathway (Storni and Bachmann, 2004). Additionally, Ria et al. (2004) have compared the response to hen egg-white lysozyme (HEL) and its reduced and carboxymethylated form (RCM-HEL) and have found that RCM-HEL induces an in vivo T-cell response that is focused on the same immunodominant determinant that characterizes the response to native HEL but that the RCM-HEL response is skewed toward the Th1 pathway. No difference in the efficiency of processing was observed between HEL and RCM-HEL. We conclude that the makeup of the T-cell epitopes of an antigen is primarily determined by the amino acid composition of the protein in the context of the peptide binding sites of MHC molecules, and not by the mechanism of delivery of the protein. These findings support the usefulness of novel immunogens, such as antigen chimeras and DNA vaccines encoding antigens with modified cell trafficking signals, as vaccine candidates, despite their differences from normal pathogen proteins in terms of antigen structure, delivery, and processing mechanisms.

The differences in the results we obtained for the three antigen preparations were largely related to the character and magnitude of the immune responses. Mice immunized with exogenous N protein plus Freund's adjuvant responded with a greatly enhanced antibody response and a high ratio of T-cell IL-4 to IFN- γ responses to the antigen peptides. In contrast, the same peptides elicited a balanced IFN- γ and IL-4 response and lower antibody responses in mice immunized with DNA vaccines. Freund's adjuvant has been associated with the preferential induction of T-cell Th2 responses (Shibaki and Katz, 2002; Yip et al., 1999; Billiau and Matthys, 2001), and different T-cell cytokine responses to the same epitope (Varga et al., 2000) have been attributed to variable expression patterns of single cells and populations that reflect transient rather than heritable differences in the expression profile (Kelso and Groves, 1997; Kelso et al., 2002). Another difference between the vaccines was the comparatively poor memory response to the N-GST protein and p-N DNA construct as compared to the p-hLAMP-N chimera construct. The limited efficacy of the conventional p-N DNA construct is consistent with many previous studies of our and other laboratories (Martins et al., 1995; Maecker et al., 1998; Oehen et al., 2000; Rush et al., 2002; Marques et al., 2003; Barros de Arruda et al., 2004). Although it is likely that there are multiple factors involved in memory T-cell differentiation (Masopust et al., 2004), we attribute the enhanced T-cell and memory responses to p-LAMP-N, at least in part, to the increased efficiency of trafficking of the LAMP-N chimera to the MHC II compartments of APCs.

The localization of the dominant T-cell responses to a cluster of overlapping peptides epitopes within N₇₆₋₁₁₄ is similar to findings from other ongoing studies of HIV-1 Gag and yellow fever virus envelope proteins where the major T-cell responses

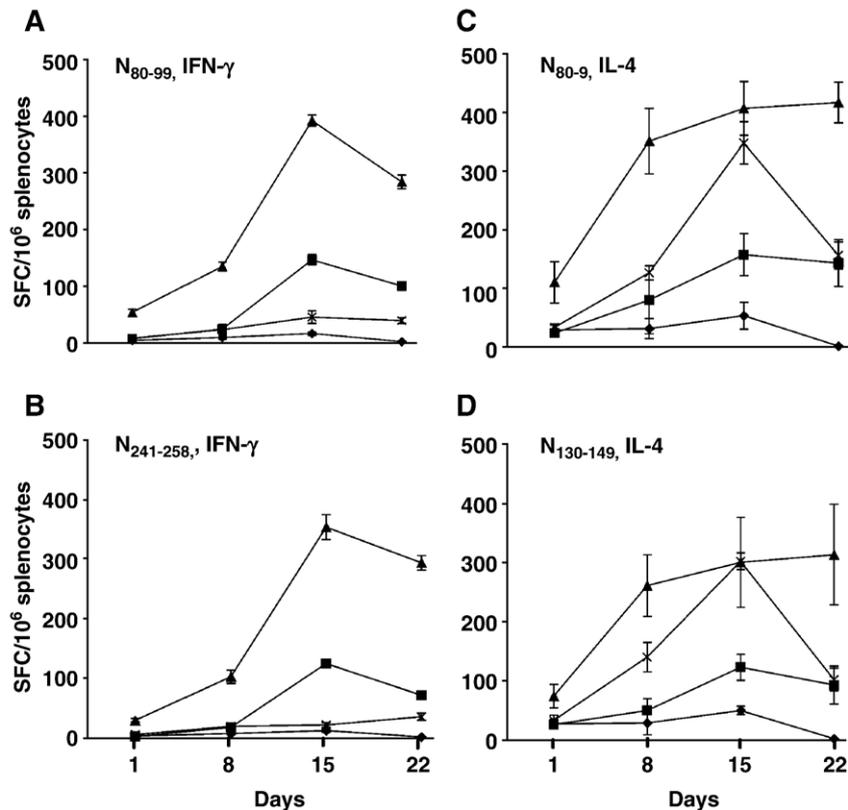


Fig. 5. Kinetics of memory IFN- γ and IL-4 ELISpot responses of N-immunized mice. Mice were immunized three times over 6 weeks with p-hLAMP (control ◆), p-N (■), p-hLAMP-N (▲), N-GST (×). At week 42, after an interval of 8 months, the memory immune response was activated by a single injection of the p-N construct. Mice were sacrificed 1 day before the final p-N injection and three times at weekly intervals thereafter (days 1, 8, 15, and 22). All assays were conducted in triplicate with cells from each of three mice from each experimental group and at each time point. ELISpot IFN- γ memory was measured in response to N₈₀₋₉₉ (A) and N₂₄₁₋₂₅₈ (B); and IL-4 in responses to N₈₀₋₉₉ (C) and N₁₃₀₋₁₄₉ (D). The p-hLAMP-N immunogen elicited the strongest IFN- γ and IL-4 ELISpot memory T-cell responses. N-GST immunogen elicited strong IL-4, but little IFN- γ response.

also involve clustered epitopes (unpublished observations). Regions of overlapping T-cell epitopes are also reported in studies of HIV-1 proteins (Shankar et al., 1996; Surman et al., 2001; Brown et al., 2003; Berzofsky et al., 1991), the outer membrane protein of *Chlamydia trachomatis* (Kim and DeMars, 2001), and in predictions of HLA T-cell epitopes (Srinivasan et al., 2004; Zhang et al., 2005). Clustered T-cell epitopes appear to be a common, perhaps ubiquitous occurrence

and, as such, may facilitate the development of epitope-based vaccines if a single or few hot spots elicit all of the required T-cell functions. The basis for such hot spots is unknown but is likely to be related in some measure to the binding affinities of the peptide sequences to MHC molecules as well as to other functional parameters that affect MHC–peptide binding to T-cell receptors during T-cell activation (Chen et al., 2005). Analysis of the nonameric MHC binding motifs of SARS N by

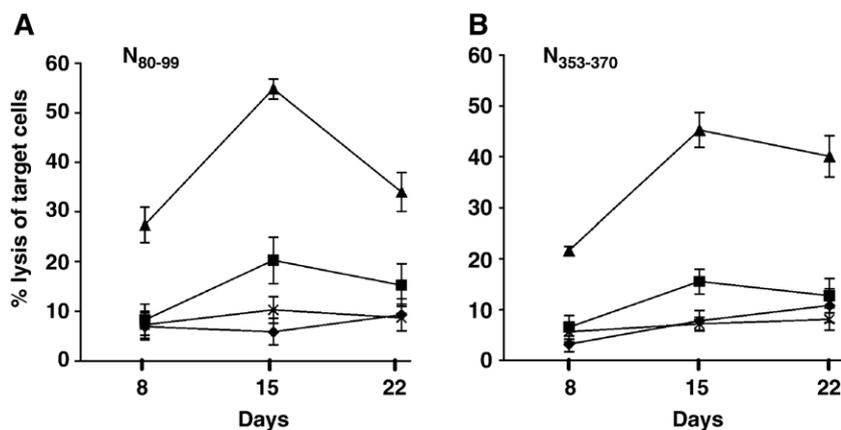


Fig. 6. Kinetics of memory CTL responses of N-immunized mice. Mice were immunized and investigated under the same protocol as described in Fig. 5 with p-hLAMP (control ◆), p-N (■), p-hLAMP-N (▲), and N-GST (×). CTL activity was assayed in responses to N₈₀₋₉₉ (A) and N₃₅₃₋₃₇₀ (B). The p-hLAMP-N immunogen elicited the strongest memory CTL responses with relatively little response of mice immunized with N-GST protein or DNA encoding unmodified N.

Table 2
Correlation of ELISpot-positive peptides and computational predictions of N-specific H2^d binding motifs

IFN- γ N peptides	PRED ^{BALB/c} H2 ^d alleles				
	H2-K ^d	H2-L ^d	H2-D ^d	I-A ^d	I-E ^d
76NTNSGPDDQIGYYRRATR ₉₃	–	–	SGPDDQIGY	–	–
80GPDDQIGYYRRATRRVRGGD ₉₉	YYRRATRRV	GPDDQIGYY	IGYYRRATR GYRRATRR	GYRRATRR	GYRRATRR
84QIGYYRRATRRVRGGDGK ₁₀₁	YYRRATRRV	–	IGYYRRATR GYRRATRR	GYRRATRR	RRVRGGDGK
92TRRVRGGDGKMKELSPRW ₁₀₉	–	–	GGDGKMKEL	–	RRVRGGDGK VRGGDGKMK GKMKELSPR
100GKMKELSPRWYFYLL ₁₁₄	ELSPRWYFY	LSPRWYFYY SPRWYFYLL	LSPRWYFYY	–	GKMKELSPR LSPRWYFYY
130GIVWVATEGALNTPKDHIGT ₁₄₉	ALNTPKDHI	VWVATEGAL	–	EGALNTPKD	WVATEGALN TEGALNTPK
241QQGQTVTKKSAEASKK ₂₅₈	–	–	–	QQGQTVTKK GQTVTKKSA VTKKSAAEA TKKSAAEAS	–
352LLNKHIDAYKTFPP ₃₆₆	LLNKHIDAY NKHIDAYKT	–	–	NKHIDAYKT	LLNKHIDAY
353LLNKHIDAYKTFPPTEPK ₃₇₀	LLNKHIDAY NKHIDAYKT	–	–	NKHIDAYKT	LLNKHIDAY

The N-specific H2^d binding motifs were analyzed by the PRED^{BALB/c} system. Each of the IFN- γ ELISpot peptides contained multiple nine amino acids binding motifs that were within the top 5% of all binding scores to the five H2^d alleles. All of the IFN- γ ELISpot peptides except N_{241–258} scored were predicted to contain class I binding motifs. Similarly, class II binding motifs were predicted in all except N_{76–93} and N_{130–149}.

a newly developed PRED^{BALB/c} computational system for predicting the probability of binding of each nine-amino-acid sequence of an antigen to the H2^d class I (H2-K^d, -L^d, -D^d) and class II (I-E^d, I-A^d) binding motifs (Zhang et al., 2005) showed that each of the SARS N peptides that elicited ELISpot-positive IFN- γ T-cell responses contained high-probability binding motifs for MHC class I and/or II alleles. There may also be protein structural effects in the selection of epitope hot spots. The three-dimensional structure of the N amino-terminal domain, N_{45–181}, of a truncated model lacking N_{1–44} and N_{182–422} has been determined by NMR spectroscopy (Huang et al., 2004b). Two of the ELISpot-positive sequences, N_{76–114} and N_{130–149}, were present on the exposed surface of this structure. The N_{76–114} T-cell hot spot sequence also contains the N_{76–101} sequence reported to comprise a B cell epitope (Huang et al., 2004a). An association between T- and B-cell epitopes has also been found for the HIV envelope; these are seen to be present in exposed loops or strands when mapped onto the crystal structure of the protein (Brown et al., 2003; Del Porto et al., 2002; Shirai et al., 1999).

Materials and methods

Cell lines

Mouse B lymphoma and monkey kidney cell lines (LB27.4 and COS-7) were obtained from ATCC (Rockville, MD). Human melanoma cell line (Mel-Juso) was obtained from German Collection of Microorganisms and Cell Cultures (Braunschweig Germany). Insect cell lines SF9 and High5 were obtained from Invitrogen. All the cell lines were maintained according to the supplier's protocols.

SARS CoV N DNA plasmids

The SARS CoV nucleocapsid (N) cDNA was prepared from a Singapore clinical isolate and purified total RNA. The 1269 bp N gene sequence was obtained by PCR with primers 5'-CGGCTAGCATGTCTGATAATGGACCCCAATC-3' and 5'-GCGGTACCTTATGCCTGAGTTGAATCAGCAG-3', having *NheI* and *KpnI* sites. The PCR product was cloned into the p43 expression vector containing AAV-ITR sequences flanking the expression elements and a CMV promoter (Kessler et al., 1996) to make the wild-type plasmid p-N. This N-sequence (without the stop codon) was amplified, and *XhoI* and *EcoRI* sites were introduced by using primers 5'-CGCTCGAGATGTCTGATAATGGACCCCAATC-3' and 5'-CGGAATTCTGCCTGAGTTGAATCAGCAGAA-3'. The p-hLAMP-N chimera vector was constructed by substitution of the HIV-gag sequence into the p-hLAMP-gag vector (Chikhlikar et al., 2004). Due to the presence of internal *NheI* or *XhoI* sites in the N sequence, the p-N and p-hLAMP-N plasmids were constructed by sequential cloning of the two fragments of the N gene.

E. coli and baculovirus N-expression systems

SARS-CoV N was amplified from the RT-PCR sequence using forward primer 5'-ACGTCAGAATTCATGTCTGATAATGGACCCCAA with an *EcoRI* site and reverse primer 5'-AGTTTAGCGGCCGCTGCCTGAGTTGAATCAGCAG with a *NotI* site. The cDNA sequence was cloned into the *EcoRI* and *NotI* sites of the pGEX6P-1 (Invitrogen, a kind gift from Dr. Roland of the Institute of Molecular and Cell Biology, Singapore) and pFastBac HT-A (Invitrogen, Carlsbad, CA) vectors. All clones were verified by sequencing.

Expression of the SARS CoV N and LAMP-N proteins

Expression of the N protein from the vaccine plasmids p-N and p-LAMP-N in the COS-7 cell line was analyzed by Western blot analysis. Cells were transfected with the SARS plasmids using Polyfect Transfection reagent (Qiagen GmbH). Cell culture and polyacrylamide gel electrophoresis were carried out as described (Chikhlikar et al., 2004). The N protein was detected by incubation with polyclonal serum from mice immunized with N-GST protein followed by horseradish-peroxidase-conjugated goat anti-mouse IgG monoclonal antibody (Pierce Biotechnology). Binding of secondary antibody was visualized with the SuperSignal West Pico Chemiluminescent Kit or DAB (Pierce Biotechnology).

Recombinant N protein expression, purification, and Western blot analysis

Recombinant N protein was generated by use of two DNA plasmid expression systems, N-GST in *E. coli* for use as an antigen for mouse immunization and N-His in a baculovirus system as an antigen for ELISA and ELISpot assays. Recombinant N protein was expressed as a GST fusion in Top10 *E. coli* cells (Invitrogen) after IPTG induction. The protein was purified by affinity chromatography with glutathione S-Sepharose beads (Amersham Pharmacia Biotech AB), and the preparation was validated by Western blot analysis using mouse monoclonal anti-GST (Santa Cruz Biotechnology) and rabbit polyclonal anti-N (Imgenex) antibodies. The Bac to Bac baculovirus expression system version C (Invitrogen) was used for N protein expression in insect cells. pFastBacHT-A-N was constructed by cloning the *EcoRI*- and *NotI*-digested SARS CoV N fragment into the pFastBacHT-A vector. N protein was purified from lysates of infected High5 cells using Ni-NTA agarose beads (Invitrogen), and the protein preparation was verified by Western blot analysis with mouse monoclonal Penta-His-antibody (Qiagen, GmbH) and anti-N (Imgenex) antibodies. Purification of the culture products of each expression system yielded fractions enriched for the 73-kDa N-GST and 55-kDa N-His proteins.

Immunofluorescent confocal and immunoelectron microscopy

The cellular localization of the expressed viral proteins in transfected mouse B lymphoma cells was studied by confocal and immunoelectron microscopy and confirmed by confocal microscopy of transfected human Mel-Juso cells. Cells were transfected with the SARS CoV plasmids (p-hLAMP-N and p-N) using Polyfect (Qiagen, GmbH). The expression of the recombinant viral proteins was visualized by the use of rabbit polyclonal anti-N (Imgenex) or mouse monoclonal anti-human LAMP1 antibody H4A3 (obtained from supernatant of hybridoma cultures prepared in our laboratory). Localization of the transgenic proteins in MHC-class-II-containing compartments and lysosomes of B cells was determined by the use of anti-mouse MHC class II antibody M5/114.15.2 (BD Biosciences) and anti-mouse LAMP1 (mLAMP1) ID4B antibody

(prepared in our laboratory). Localization of the viral proteins in the MHC class II-containing compartments of Mel-Juso cells was analyzed with anti-HLA-DR, DP, and DQ antibodies (BD Pharmingen) and rabbit anti-N antibody (IMG-549; Imgenex).

Mouse immunization

Three immunization protocols were carried out: (1) three groups (p-hLAMP control, p-N, and p-hLAMP-N immunogens) of 5 female (6 to 8 weeks old) BALB/c mice per group were immunized subcutaneously at the base of the tail with 50 μ g of specified endotoxin-free DNA plasmid diluted in PBS on days 1, 22, 43, 64, and 100. Blood samples were collected by phlebotomy from the tail vein on days 0, 21, 42, 63, 84, 99, and 108, and the animals were sacrificed on day 108, 8 days after the last immunization. (2) Another group was immunized intraperitoneally with 10 μ g of N-GST protein emulsified with CFA followed by two booster immunizations 3 weeks apart with N-GST protein emulsified with IFA. Blood samples were collected as outlined above on days 0, 21, and 42, and the animals were sacrificed on day 50, 8 days after the last immunization. Protocols 1 and 2 were carried out twice with five mice per group each time. (3) An additional protocol was carried out for analysis of memory responses: four groups of 16 mice each (p-hLAMP control, p-N, p-hLAMP-N, and N-GST immunogens) were immunized as outlined above, but with only three injections over 6 weeks. At week 42, after an interval of 36 weeks, the memory immune response was activated by a single injection of the p-N construct with p-hLAMP as the negative control. Three mice of each group were sacrificed 1 day before the final p-N injection and at weekly intervals for 3 weeks thereafter. These experiments were performed as approved by the Johns Hopkins University Animal Care and Use Committee under Protocol Number MO04M178.

ELISA for N-specific antibody analysis

Anti-SARS CoV N IgG antibodies in the serum from each mouse were assayed by ELISA for seroconversion at a dilution of 1:300, and the total IgG titers of 3-fold serial dilutions, starting from 1:100, were determined by standard quantitative ELISA. In brief, ELISA plates (96-well, MaxiSorb F96; Nunc Inc.) were coated overnight at 4 °C with 1 μ g/ml recombinant N-His protein in PBS followed by blocking with ELISA buffer (PBS containing 2.5% milk and 0.05% Tween 20). After washing, appropriately diluted mouse serum was added and incubated for 2 h at room temperature. Total IgG was detected with the Mouse Extravidin alkaline phosphatase staining kit (Sigma).

ELISpot analysis of antigen-activated T cells

The response of antigen-specific T cells from immunized mice was measured by the use of IFN- γ and IL-4 ELISpot sets (BD Pharmingen) according to the manufacturer's protocol. Splenocytes were stimulated with 5 μ g/ml of recombinant N-

His (baculovirus-produced) or 10 µg/ml of the overlapping SARS CoV synthetic peptides from three sources (NIH AIDS Research and Reference Reagent Program, Rockville, MD; SynPep Corp, Dublin, CA; the Johns Hopkins Biochemistry Core Facility). Negative control stimulation produced less than five spots per well in >90% of experiments. The average number of spots in the negative control wells was 1.67 ± 1.87 . Each experiment was repeated at least twice on different groups of mice from two immunizations.

Cytotoxic T-lymphocyte assay

CTL assays were performed using the Cytotox 96 non-radioactive cytotoxicity assay kit (Promega Corporation, USA). The pooled splenocytes (from two mice in the N-GST group and three mice in each of the other groups) obtained at each time point (days 1, 8, 15, and 22) after memory recall with pN were stimulated *in vitro* with each of the two peptides, N_{80–99} and N_{353–370}, at a final concentration of 10 µg/ml for 4 days. SP2/O target cells were pulsed overnight separately with 10 µg/ml of each of the two peptides, washed two times, resuspended in RPMI supplemented with 2% FCS, and seeded onto a 96-well U-bottom plate (1×10^4 cells/well in 50 µl of culture medium) with an equal volume of pooled, *in vitro*-stimulated splenocytes from recalled mice (effector cells) at different effector/target ratios, 20:1, 40:1, and 80:1. All incubations were done in quadruplicate. The negative control contained an equal concentration of unrelated peptide, and controls for effector cell spontaneous release, target spontaneous release, and target maximum release were included in the assays. All the values were computed as the mean \pm standard deviations of the four replicate assays after subtracting the average of the values obtained for the negative controls. The use of overlapping peptides for antiviral CD8 and CD4 T-cell responses has been described (Maecker et al., 2001; Draenert et al., 2003).

Prediction of T-cell binding motifs

BALB/c mouse H2^d T-cell binding motifs of the 60 overlapping N peptides were predicted by use of the PRED^{BALB/c} class I (H2-K^d, -L^d, -D^d) and class II (I-E^d, -A^d) models (Zhang et al., 2005). The PRED^{BALB/c} system scores the binding index of all immunogen nine-amino-acid sequences to H2^d molecules, based on quantitative matrices.

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