Immune responses to T-cell epitopes of SARS CoV-N protein are enhanced by N immunization with a chimera of lysosome-associated membrane protein

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In our previous study by Gupta et al, dominant T-cell epitopes of SARS CoV-N(N) protein were predicted by software. The spectrum of interferon (IFN)-γ responses of Balb/c mice immunized against two different forms of SARS CoV-N plasmid was then analyzed. A cluster of dominant T-cell epitopes of SARS CoV-N protein was found in the N-terminus (amino acids 76–114). On the basis of this study, four different plasmids were constructed: (i) DNA encoding the unmodified N (p-N) or N70–122 (p-N70–122) as an endogenous cytoplasmic protein or (ii) DNA encoding a lysosome-associated membrane protein (LAMP) chimera with N (p-LAMP/N) or N70–122 (p-LAMP/N70–122). The immune responses of mice to these four constructs were evaluated. The results showed marked differences in the responses of the immunized mice. A single priming immunization with the p-LAMP/N construct was sufficient to elicit an antibody response. Enzyme-linked immunospot (ELISpot) assay indicated that p-LAMP/N70–122 and p-LAMP/N plasmids both elicited a greater IFN-γ response than p-N. p-N and p-N70–122 constructs induced low or undetectable levels of cytokine secretion. We also found that the p-LAMP/N70–122 construct promoted a long-lasting T-cell memory response without an additional boost 6 months after three immunizations. These findings show that DNA vaccines, even epitope-based DNA vaccines using LAMP as chimera, can elicit both humoral and cellular immune responses.

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Introduction

Endogenous antigens encoded by conventional naked DNA plasmids are processed by antigen-presenting cells and presented only to CD8+ T cells through the formation of peptide/major histocompatibility complex class I (MHC I) complexes. Therefore, CD4+ T cells cannot be effectively activated in this model. CD4+ T cell activation is vital for the efficient priming of CD8+ T cells, B lymphocytes and long-term immune memory.1–6 A weak point in the current theory is that DNA vaccines, when encoding a cytoplasmic protein, usually fail to elicit a sufficient, if any, CD4+ T response, whereas MHC I–cytotoxic T-lymphocyte (CTL) responses are usually preferable. This preference usually leads to the inability of the host immune system to mount greater immune responses and to maintain long-term memory.

The lysosome is one of the most important components of the multilaminar vesicular compartment (MHC class II-processing compartment) in the exogenous antigen-processing pathway.7–9 Lysosome-associated membrane protein (LAMP) can target and bind the endosome/lysosome through the LAMP transmembrane/cytoplasmic domain. The luminal domain of LAMP is then integrated into the lysosome.10–17 Consequently, one strategy commonly adopted by our group18–25 is to insert the target DNA into LAMP as a LAMP/antigen chimera. In addition, many other laboratories have reported that LAMP targeting could greatly enhance the immune response against a number of antigens.26–38 It has been postulated that DNA plasmids encoding endogenous antigen could take advantage of LAMP and be carried directly into MHC class II-processing compartment, where the fusion protein of interest could be digested and presented by MHC II molecules. This strategy could thus boost the CD4+ T-lymphocyte response and, in turn, enhance the immune responses mentioned above.
Our previous work showed that the LAMP–SARS Coronavirus nucleocapsid construct (p-LAMP/N) elicits the strongest T-cell responses when compared with the SARS CoV-N alone (p-N) or a glutathione S-transferase–N fusion protein. A dominant epitope cluster appeared to be located within the N terminus of SARS CoV-N. Hence, we were interested to find out whether a sequence containing these epitopes only would suffice to elicit a T-cell response and, if not, whether it was possible to make a LAMP fusion construct to enhance the immune response. In this paper, we will explore the above-mentioned possibilities.

Results

Expression of N, LAMP/N, N\textsubscript{70–122} and LAMP/N\textsubscript{70–122} in transfected cells

Several DNA plasmids encoding N, N\textsubscript{70–122} or LAMP protein chimeras were constructed in the p43 vector backbones to evaluate protein expression in transfected cells. Expression of the protein encoded by the DNA plasmid constructs in transfected COS-7 or 293 cells was confirmed by fluorescence microscopy and flow cytometry for proteins containing the enhanced green fluorescent protein (EGFP) tag, as well as by western blot.

The levels of EGFP-tagged N\textsubscript{70–122} and LAMP/N\textsubscript{70–122} expression in transfected 293 or COS-7 cells were analyzed by fluorescence microscopy (Figure 1a) and flow cytometry (Figure 1b). No significant difference in the fluorescence intensity of the transfected cells was observed, suggesting similar synthesis and translation rates for the two EGFP-tagged molecules.

Western blot using the anti-SARS-N\textsubscript{70–169} polyclonal antibody confirmed the presence of the approximately 160 kDa LAMP/N protein and 45 kDa N protein at comparable levels in transfected 293 cells. Protein degradation of N was detected in the LAMP chimera-transfected cells. LAMP/N\textsubscript{70–122} was present at about twofold greater concentrations than the modified N\textsubscript{70–122} (Figure 1c) by Bio-Rad ChemiDoc scanning (Bio-Rad, Hercules, CA, USA).

Greater concentrations of the LAMP chimera forms of proteins have been observed frequently in cell extracts, presumably because of the increased expression of the associated LAMP, a major cellular structural protein, or because of decreased proteolytic degradation as a consequence of the association with the heavily glycosylated and protease-resistant LAMP.

Antibody responses to repeated immunizations with the SARS CoV-N DNA constructs

Mice were immunized three times (on day 1 and by boosting immunizations on days 21 and 42) by subcutaneous injection of the DNA plasmid. An initial evaluation of the immune response was conducted by measuring antibody binding to the intact N protein 2 weeks after each immunization. An antibody response to LAMP/N immunogen was detected after the first immunization, and strong responses to both N and LAMP/N were detected after the second immunization. A low antibody response to LAMP/N\textsubscript{70–122} was observed after the third immunization, compared with both LAMP/N and N-immunized mice. However, the response of mice immunized with LAMP/N\textsubscript{70–122} was considerably greater than the response of those immunized with N\textsubscript{70–122}, which did not differ from either the immunogen control group or the normal mouse serum (Figure 2).

Peptide-specific IFN-γ, IL-2 and IL-4 ELISpot responses to a dominant cluster of T-cell epitopes

We previously reported that the major interferon (IFN)-γ T-cell response of mice immunized with N DNA constructs was to a cluster of N overlapping peptides encompassing amino acids 76–114 from the N-amino terminus, with an approximately twofold greater response to LAMP/N compared with N. Sequential studies of this epitope cluster as a potential epitope-based DNA vaccine have shown a remarkable level of T-cell activation by the fragment constructed in the form of a LAMP chimera. Mice were immunized with constructs encoding the intact N and the N\textsubscript{70–122} sequence, both as unmodified sequences and as LAMP chimeras. The experimental protocol, as described in Materials and methods, included immunization with p-N and p-LAMP/N as positive controls and, for comparison with previous experiments, with p-N\textsubscript{70–122} and p-LAMP/N\textsubscript{70–122} for analysis of the dominant epitope cluster as an N antigen and with a p-LAMP/dengue-env construct as a negative-control immunogen.

Enzyme-linked immunospot (ELISpot) assay responses after two immunizations (on day 40) were analyzed with overlapping peptides spanning the N epitope cluster region, amino acids 70–122, and with a yellow fever virus peptide as a negative-control peptide. The IFN-γ response results for the positive-control immunizations with p-N and p-LAMP/N were similar to those reported earlier, with strong T-cell responses to LAMP/N immunization that were approximately twofold greater than N immunization (Figure 3a-1). It is noted, however, that a response to p-N\textsubscript{70–122} lacking the LAMP sequences was not detected and was equivalent to that of the negative control (p-LAMP/den-env). In contrast, strong responses to the p-LAMP/N\textsubscript{70–122} construct were detected, comparable to immunization with the intact N protein. Moreover, the p-LAMP/N\textsubscript{70–122} immunized mice showed strong interleukin (IL)-2 and IL-4 responses, similar to those elicited by p-LAMP/N, whereas very little response was observed in the p-N and p-N\textsubscript{70–122}immunized mice (Figures 3a-2 and a-3).

Enhanced IFN-γ, IL-2, IL-4 and IL-10 T-cell responses after three times of LAMP/N\textsubscript{70–122} or LAMP/N immunizations

An additional (second boost) immunization on day 42 with p-LAMP/N and p-LAMP/N\textsubscript{70–122} resulted in dramatic cytokine production measured on day 60 (Figure 3b), with the most dramatic results observed for IFN-γ, IL-2, IL-4 and IL-10. Again, almost no IFN-γ, IL-2, IL-4 or IL-10 response was displayed by the p-N\textsubscript{70–122} immunized mice, and very little response other than IFN-γ was detected for the p-N-immunized mice. In addition, there was a marked broadening of the enhanced response to the LAMP chimera constructs that included all of the peptides encompassing the cluster of
epitopes, and this was presumably because of the bystander activation.

**The requirement for LAMP chimera constructs to induce memory T-cell responses**

Memory T-cell responses were measured by ELISpot at week 30 in mice treated with the same initial immunization protocol with or without an additional boost injection at week 27. After the boost injection at week 27, strong memory T-cell IFN-γ responses were induced by p-LAMP/N, p-LAMP/N70-122 and p-N and, in contrast, no significant response to p-N70-122 was observed (Figure 4a-2). Without the boost immunization, there were relatively weak but significant memory responses to the LAMP chimera constructs, compared with no response to constructs lacking LAMP (Figure 4a-1).

The splenocytes of immunized mice lacking an additional boost injection at week 30 were cultured in medium alone or with a pool of overlapping peptides spanning the SARS-N epitope cluster region (amino acids 66–128) for 2 or 5 days. The cultured supernatants were respectively collected. The cytokine levels in the supernatant were then determined by enzyme-linked immunosorbent assay (ELISA). At 2 days, greater memory T-cell cytokine secretion, including IFN-γ, IL-4, IL-5 and IL-10, was observed with the p-LAMP/N and p-LAMP/N70-122 constructs in contrast to an almost undetectable response to p-N70-122 and p-N. The strongest memory T-cell cytokine secretion was observed

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**Figure 1** Validation of SARS-N and N70-122 protein expression. 293 or COS-7 cell lines were transfected with different DNA plasmids. (a) and (b) Enhanced green fluorescent protein (EGFP) expression was directly observed by Fluorescence microscopy and flow cytometry. (c) Western blot analysis of transfected 293 cells blotted with anti-N70-122 polyclonal antibody. The positions of the protein markers in kDa are indicated on the right. LAMP, lysosome-associated membrane protein.
Figure 2: Humoral immune responses. (a–c) Mice were injected on days 1, 21 and 42 with 50 μg of DNA plasmid. Samples were collected 2 weeks after each immunization. Pooled serum from individual groups of mice was serially diluted, and SARS-N-specific total IgG was measured by enzyme-linked immunosorbent assay. LAMP, lysosome-associated membrane protein.

Figure 3: The lysosome-associated membrane protein (LAMP) chimera enhances the antigen-specific cytokine responses. Mice were injected twice (a1–a3) and three times (b1–b4) with 50 μg of DNA plasmid. Antigen-specific splenocyte cytokine secretions were detected by enzyme-linked immunospot assay. The splenocytes were stimulated with overlapping peptides spanning the SARS-N epitope cluster region, amino acids 70–122, and with a yellow fever virus (YFV) peptide as a negative control. Each result was performed in three times individual experiment.
in response to the p-LAMP/N and p-LAMP/N_{70-122} constructs at 5 days (Figure 4b).

**Discussion**

Endogenous antigens are processed in the cytoplasm and initiate CTL activation. In contrast, exogenous antigens are degraded in the lysosome of antigen-presenting cells and induce antibody-mediated humoral immune responses and CD4+ T-cell activation and secretion of cytokines to facilitate CTL activation and differentiation. Therefore, the achievement of a desired response by controlling the delivery pathways is indispensable in vaccine development and immunotherapy. In addition, the effective activation of CD4+ T cells is critical for generating adaptive immune responses. The studies discussed above used a recently developed approach that provides an additional necessary protein element required for antigen trafficking to vesicular lysosomal sites where MHC II antigen presentation takes place. As shown in the results, the DNA vaccine of SARS-CoV-N alone definitely elicited the host immune response, and this was most evident for B cells. However, stronger T-cell responses were observed when LAMP fusion DNA vaccines were applied. This is consistent with previous
studies in which HIV Gag-LAMP elicited superior antibody and CD4+ and CD8+ cellular responses.\(^\text{18}\) This increase in the CD8+ T-cell response could be attributed to the increased response of CD4+ T cells. This study, together with previous studies, provides indirect evidence that the trafficking of DNA-encoded antigens to the LAMP endosomal/lysosomal vesicular compartments is an important factor affecting the function of genetic vaccines. Whatever the mechanism may be, the effect is evident. In addition to stronger T-cell responses (Figure 3), stronger long-term memory T-cell responses (Figure 4) were also observed, even with the p-LAMP/N\(_{70–122}\) construct, whereas almost no response was detected to the p-N construct. These results reveal the special advantage of LAMP fusion DNA vaccines, which have a much longer effect than conventional DNA vaccines.

Another finding of this study confirmed our initial prediction that the T-cell dominant epitope cluster of SARS CoV-N resides mainly in the N-terminal 76–114 amino acids of this protein (Figure 5). It is interesting that even though this dominant epitope cluster resides in this area, the DNA SARS CoV-N\(_{70–122}\) vaccine alone failed to elicit immune responses in either B cells or T cells. However, when the fragment was fused to LAMP, dramatic immune responses were observed. The extent of the T-cell response was comparable to that observed with p-LAMP/N, and was stronger than that detected with p-N. Weaker B cell responses were also evident (Figure 2). There are some possible explanations for this result. The major B cell epitope cluster may not be located within the N terminus of SARS CoV-N. This hypothesis is supported by the fact that the p-LAMP/N construct elicited a B-cell response equivalent to the p-N construct and to the findings reported by Zhao \textit{et al}.\(^\text{40}\)

Taken together, this study not only showed that the use of LAMP as a chimera can elicit both humoral and cellular immune responses, even with an epitope-based cluster DNA vaccine, but it also emphasized the significance of targeting DNA-encoded vaccine antigens to the MHC II-processing compartments for induction of long-term immunological memory. These findings are of great value in understanding anti-SARS-CoV immunity and also in designing epitope-based DNA vaccines against the disease.

**Materials and methods**

**Plasmids**

The SARS CoV-N cDNA was prepared from a Singapore clinical isolate (GenBank ID, AY307163), as previously described.\(^\text{23}\) The total N 1–1269 bp sequence and a 208–366 bp fragment were cloned into the p43 vector,\(^\text{41}\) which contains the adeno-associated virus inverted terminal repeat sequences flanking the expression elements (cytomegalovirus promoter and bovine growth hormone polyadenylation signal). The complete N gene fragment was amplified by PCR using the following primers:

\[
\begin{align*}
5' - CGGCTAGCACATGCTCAGTATAATGGAACCCATCATC-3' \\
5' - GGTACCTATATGCCCTAGTTGAATCGACAG-3' \\
5' - CCGCTAGAGATCAGTCTGATAATGGACCCGAAATCT-3' \\
5' - CGGAATTCTGCCTAGTTGAATCGACAG-3'
\end{align*}
\]

The 208–366 bp gene region was amplified using the primers:

\[
\begin{align*}
5' - GGGAATCCATGGCCAGGCGTTCATAATCCAACACCAT-3' \\
5' - CCGTTACCTTAAGTAGTGAAGCTTCTGGGACCAGG-3' \\
5' - CCGCTAGAGATCAGTCTGATAATGGACCCGAAATCT-3' \\
5' - CCGAATTCTGCCTAGTTGAATCGACAG-3'
\end{align*}
\]

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\begin{align*}
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5' - CCGTTACCTTAAGTAGTGAAGCTTCTGGGACCAGG-3' \\
5' - CCGCTAGAGATCAGTCTGATAATGGACCCGAAATCT-3' \\
5' - CCGAATTCTGCCTAGTTGAATCGACAG-3'
\end{align*}
\]

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5' - GGGAATCCATGGCCAGGCGTTCATAATCCAACACCAT-3' \\
5' - CCGTTACCTTAAGTAGTGAAGCTTCTGGGACCAGG-3' \\
5' - CCGCTAGAGATCAGTCTGATAATGGACCCGAAATCT-3' \\
5' - CCGAATTCTGCCTAGTTGAATCGACAG-3'
\end{align*}
\]

The amplified fragments of native N and N\(_{70–122}\) were inserted into the Nhel and KpnI sites of the p43 vector and termed p-N and p-N\(_{70–122}\) respectively. The LAMP chimera constructs, p-LAMP/N and p-LAMP/N\(_{70–122}\),
Protein expression analysis

Enhanced green fluorescent protein-tagged N70-122 and LAMP/N70-122 protein expressions were studied in human 293 and COS-7 monkey kidney cell lines. Cells were seeded in 6-well plates (1 × 10^5 cells per well or 5 × 10^5 cells per well) during log-phase growth and transfected with the EGFP-labeled N70-122 and LAMP chimera plasmid DNA (4 μg) using FuGENE 6 transfection reagent (Roche, Indianapolis, IN, USA), according to the manufacturer’s instructions. EGFP expression was observed 24–48 h after transfection by fluorescence microscopy and flow cytometry.

Western blot analysis of protein expression was analyzed using 293 cells. Cells were seeded in 6-well plates (2 × 10^5 cells per well) and transfected with plasmid DNA lacking the EGFP tag using the same transfection reagent. The cells were harvested 48 h after transfection and disrupted with CelLytic M Cell Lysis Transfection reagent. The cells were harvested 48 h after transfection by fluorescence microscopy and flow cytometry.

Animals and immunization

Five groups of 6–8-week-old female Balb/c mice (Charles Rivers, Wilmington, MA, USA; each group contained 30 mice) were immunized subcutaneously at the base of the tail with 50 μg of the specified endotoxin-free DNA plasmid (p-N, p-LAMP/N, p-N70-122, p-LAMP/N70-122 and p-LAMP/den-env as an immunogen control) diluted in phosphate-buffered saline (PBS). The mice were boosted twice every 3 weeks with the same plasmid. Blood samples were collected by tail vein bleeding of the immunized mice 2 weeks after each immunization. The immunized mice were killed on day 40 or 60.

Furthermore, to analyze the immune recall response, five groups of mice (each group contained 10 mice) were immunized as described above. After an interval of 21 weeks (at week 27), the immunized mice received an additional boost with the same DNA plasmid or with PBS alone. The immunized mice were killed after a 3-week interval (Figure 7).

Antibody determination in vaccinated mice

The 96-well ELISA plates (Nunc, Rochester, NY, USA) were coated with recombinant N protein (NIH Biodefense and Emerging Infections Research Resources Repository, NIAID). The protein was diluted in 0.1 M sodium carbonate–bicarbonate buffer (PH 9.5) at a concentration of 5 μg ml⁻¹, and 100 μl was added to each well and incubated overnight at 4 °C. The coating solution was then discarded and the plates were washed three times with PBS containing 0.05% Tween-20 washing buffer (PBST). Immunized serum samples were prepared in eight (threefold) serial dilutions in PBST with 0.1% bovine serum albumin starting at 1:100. One hundred microliters of each dilution was added to each well and the plates were incubated for 2 h at 37 °C. The serum antibody was captured by the coated N protein and detected by incubation with 100 μl of horseradish peroxidase-conjugated goat anti-mouse IgG antibody (1:1000) for 1 h at 37 °C. Color was developed with Turbo tetramethylbenzidine substrate solution (BD PharMingen, San Diego, CA, USA) by incubation for 15 min at room temperature. The reaction was stopped...
by the addition of 50 μl of 2 M sulfuric acid and the absorbance was measured at 450 nm in a TECAN microplate reader (SafireII, San Jose, CA, USA).

Preparation of splenocytes for assaying the T-cell-mediated cellular immune responses of immunized mice

Single cell suspensions depleted of red blood cells were prepared from freshly isolated immunized mouse splenocytes in culture medium (RPMI-1640 medium supplemented with 5% v/v fetal bovine serum, 100 units ml⁻¹ penicillin/streptomycin, 2 mM l-glutamine, 50 μM 2-mercaptoethanol, and 0.01 M HEPES buffer). Splenocytes were counted and resuspended at 1 × 10⁶ cells ml⁻¹ in culture medium for T-cell-mediated assays.

ELISpot assays

Cellular immune responses to N, N₇₀₋₁₂₂ and LAMP chimeras were assessed by multiple cytokine ELISpot assays according to the manufacturer’s protocol (BD Pharmingen). Briefly, ELISpot plates were coated overnight at 4 °C with cytokine-specific capture antibody (rat anti-mouse) diluted in sterile PBS at 5 μg ml⁻¹. After blocking for 2 h with RPMI-1640 (Gibco, Invitrogen Co., Carlsbad, CA, USA) containing 10% fetal bovine serum at room temperature, 1 × 10⁶ splenocytes per well were stimulated with the overlapping N synthetic peptides at a final concentration of 10 μg ml⁻¹ (Sin-pep, NIH Biodefense and Emerging Infections Research Resources Repository, NIAID) for 16–40 h at 37 °C in 5% CO₂ incubator. Negative controls were medium alone or a yellow fever virus peptide, respectively. Con A (5 μg ml⁻¹) was used as a positive control. The plates were washed with H₂O and PBST and incubated for 2 h at room temperature with 2 μg ml⁻¹ of the relevant biotinylated rat anti-mouse cytokine antibody. After washing with PBST, the plates were incubated with streptavidin–horseradish peroxidase at a 1:100 dilution for 1 h. The reaction was developed with AEC substrate (Calbiochem, San Diego, CA, USA) and was stopped by washing with water. The plates were air dried and the spots were visualized. The plates were counted and the frequencies of the cytokine-producing cells were calculated by subtracting the number of cytokines in culture medium alone or with a final concentration of 20 μg ml⁻¹ of the overlapping peptide pool (N₆₀₋₁₂₂). After incubation for 2 or 5 days at 37 °C in 5% CO₂, the culture supernatants were collected for detection of secreted cytokines as measured by OptEIA ELISA kits (BD Pharmingen).

Cytokine production by immunized splenocytes assessed by ELISA analysis

Single splenocyte suspensions from immunized mice were prepared as above, and 1 × 10⁶ splenocytes per ml per well were cultured in a 12-well plate (Nunc.) with medium alone or with a final concentration of 20 μg ml⁻¹ of the overlapping peptide pool (N₆₀₋₁₂₂). After incubation for 2 or 5 days at 37 °C in 5% CO₂, the culture supernatants were collected for detection of secreted cytokines as measured by OptEIA ELISA kits (BD Pharmingen).

Statistical analyses

The experimental data were obtained from two completely independent mouse immunizations and two different libraries of synthetic peptides. All ELISpot data are expressed as means ± s.d. All graphs and unpaired t-test analyses were generated using GraphPad Prism Version 4.0a for Macintosh (GraphPad software, San Diego, CA, USA).

Conflict of interest

The authors declare no conflict of interest.

Abbreviations

SARS, severe acute respiratory syndrome; SARS CoV, SARS-associated coronavirus; N, nucleocapsid protein; LAMP, lysosome-associated membrane protein; ELISpot, enzyme-linked immunospot assay; MHC, major histocompatibility complex; p-N, p43-SARS CoV-N construct; p-LAMP/N, p43-LAMP SARS CoV-N construct; p-N₇₀₋₁₂₂, p43-SARS CoV-N₇₀₋₁₂₂ epitopes cluster construct; p-LAMP/N₇₀₋₁₂₂, p43-LAMP SARS CoV-N₇₀₋₁₂₂ epitopes cluster construct; p-LAMP/den-env, p43-LAMP Dengue virus envelope construct.

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