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Investigation of immunogenic T-cell epitopes in SARS virus nucleocapsid protein and their role in the prevention and treatment of SARS infection

Key Messages

1. A novel HLA-A2.1–specific SARS coronavirus (SARS-CoV) nucleocapsid (N) protein epitope (N220-N228 LALLLLDRL) able to activate cytotoxic T cells in vitro has been identified.
2. When used with a single-chain-trimer system, the SARS-CoV N protein epitope (N220-N228 LALLLLDRL) can stimulate a cytotoxic T-cell response against N-protein expressing cells in the HLA-A2.1K^b transgenic mouse model.

Introduction

The investigation of immunity to the SARS coronavirus (SARS-CoV) has attracted worldwide attention since the 2003 SARS outbreak. Previous studies have suggested that HLA-A*0201 restricted SARS S- and nucleocapsid (N)-protein peptides can trigger specific human cytotoxic T-cell responses against SARS in vitro.[1] In this study, we have identified a novel HLA-A*0201 restricted epitope, N220 (LALLLLDRL), of the SARS-CoV N-protein using bioinformatic analysis. The N-protein peptide N220 has a high binding affinity for human major histocompatibility complex (MHC) class I in T2 cells, and is capable of activating cytotoxic T cells in human peripheral blood mononuclear cells (PBMCs). The possibility of combining the N220 peptide sequence with a single-chain-trimer (SCT) as a deoxyribonucleic acid (DNA) vaccine has been investigated in HLA-A2.1K^b transgenic mice. Cytotoxicity assays have shown that the T-cells obtained from the vaccinated animals were able to kill the N-protein expressing cells. They achieved a cytotoxicity level of 86% in an effector cells/target cells ratio of 81:1 one week after the last vaccination, which is significantly higher than that achieved by other N-protein peptides previously described. The novel immunogenic N-protein peptide in our study provides valuable information for designing a therapeutic SARS vaccine.

Materials and methods

The objective of this study was to identify immunogenic N-protein peptides that can serve as cytotoxic T-cell epitopes in SARS vaccines. This study was conducted from February 2005 to January 2007.

The SARS N-protein peptide sequence for human MHC class I binding was searched using a human leukocyte antigen (HLA) peptide binding prediction program, SYFPEITHI (http://www.syfpeithi.de). Seven 9-amino acid peptides with high scores for human MHC class I binding (N139-147 ALNTPKDHI, N160-168 LQLPQGTTL, N220-228 LALLLLDRL, N223-231 LLLDRLNQL, N227-235 RLNQLESKY, N317-325 GMSRIGMEV, N352-360 ILLNKHID) were synthesised.

The selected N-protein peptides were initially tested for their ability to bind to human MHC class I molecules and stimulate an immune response in vitro. The T2 cells were pulsed with each of the N-protein peptides in the presence of human β_2-microglobulin (Sigma) and were stained with a mouse anti-human HLA-A2 antibody (BB7.2) and a goat anti-mouse IgG FITC antibody (Zymed). They were then subjected to flow cytometry to measure the level of MHC class I/peptide complexes formed. For in-vitro vaccination, human HLA-A*0201 positive CD8+ T cells purified from PBMCs were subjected to autologous dendritic cell mediated T-cell activation. The N-protein peptide-loaded mature dendritic cells were co-cultured with the purified CD8+ T cells for T-cell stimulation. The stimulation was repeated once a week. A total of three T-cell stimulations with the peptide-loaded dendritic cells were conducted. T-cell activation was investigated using an IFN-γ ELISPOT assay.

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The DNA sequence of the SCT display system containing the human β_2-microglobulin and the chimeric MHC heavy chain,[2,3] was synthesised by connecting the mouse β_2-microglobulin cDNA, human HLA-A*0201 α-1 cDNA, HLA-A*0201 α-2 cDNA and the mouse H2-K^b α-3 domain cDNA together using an overlapping polymerase chain reaction (PCR) to construct an *MHK* gene (M=mouse β_2-microglobulin, H=human HLA-A*0201 α-1 and α-2, K=mouse H2-K^b α-3). The DNA fragments encoding the selected N-protein peptides were then linked to the N-terminal of the *MHK* gene by PCR using primers with the corresponding DNA sequence at the 5' region. The constructed SCT-DNA fragments were then cloned into pVAX1 (Invitrogen) to construct N-protein peptide expressing plasmids, N220MHKpVAX1, N223MHKpVAX1, N227MHKpVAX1, and N317MHKpVAX1 for vaccination purposes. OVAMHKpVAX1 was also constructed as a control plasmid expressing a SIINFEKL peptide of the ovalbumin (OVA257). The DNA plasmids were delivered into the abdominal region of 6-to-8 weeks old HLA-A2.1K^b transgenic mice (Mutant Mouse Regional Resource Centers, USA) with a Helios Gene Gun (Bio-Rad) with a discharge pressure of 400 psi. Each mouse was given 1 μg DNA each time for a total of three vaccinations each separated by a 3-week interval.

To investigate the cell-mediated cytotoxic response triggered by the SCT-DNA vaccine, mice were sacrificed 1 week after the last vaccination. The splenocytes were cultured in the presence of the corresponding target peptides and interleukin-2 (Peprotech) for 3 days. Spleen T cells were then harvested and used as effector cells in a cytotoxicity assay. The N-protein transduced N/E6E7/2.1K^b cells were used as target cells. In the cytotoxicity assay, the effector cells and the target cells were co-cultured in different ratios. After 5 hours of incubation, the culture medium was collected for further analysis using a lactate dehydrogenase (LDH) cytotoxicity detection kit (Roche) according to the procedures stated by the manufacturer.

Results and discussion

The objective of this study was to identify immunogenic N-protein peptides that can serve as cytotoxic T-cell epitopes in SARS vaccines. A peptide sequence useful for inducing the cytotoxic T-cell response should be presented as an endogenous peptide epitope through proteasome digestion and have a high binding affinity for human MHC class I molecules. A bioinformatics analysis was employed to search for the most immunogenic peptide sequences in the SARS N-protein. To investigate whether the peptide predicted by the computer program had high binding affinity with human MHC, a T2-cell binding assay was performed to assess the peptide's binding affinity to the empty MHC class I molecules on the cell surface and to stabilise the MHC class I-peptide complex formed. The results demonstrated that the novel peptide identified, N220, has a high binding affinity for the human MHC class I molecule HLA-A2.1, comparable to the N223 peptide and significantly higher than that of the N227 and N317 peptides previously described (Fig 1). T-cell activation was assessed using ELISPOT. T-cells primed with N223 (LLLDRLNQL) and N220 (LALLLLDRL) produced the highest number of spots—six to seven times higher than the number produced by the irrelevant flu peptide primed T-cells and significantly higher than those produced by the other selected N-protein peptides (Fig 2). The level of IFN-γ produced from the N220 primed T cells was comparable to that produced by the

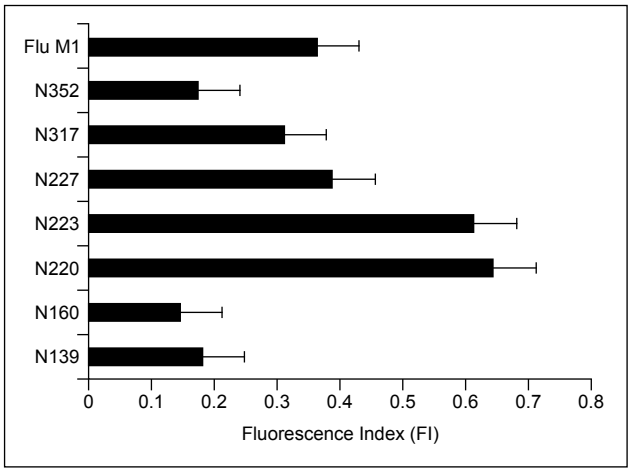

Fig 1. Comparison of the binding affinity of N-protein peptides with T2-cells
The N-protein peptides were incubated with the T2 cells in the presence of β_2-microglobulin and the stabilised MHC-class I complexes were detected using an antibody (BB7.2) via flow cytometry. The level of complexes formed is presented as the fluorescence index: [MFI(T2+peptide)/MFI(T2 only)] - 1. The flu M1 peptide (GILGFVFTL) was used as a positive control. Results represent the mean ± standard deviation (n=5)

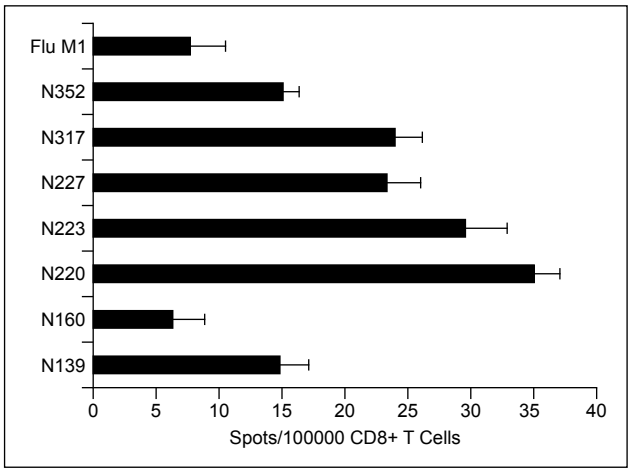

Fig 2. Activation of human T-cell in IFN-γ ELISPOT assay
CD8+ T cells primed with N-protein peptide–loaded dendritic cells were cultured with recombinant N protein–loaded autologous B cells for one day and the secretion of IFN-γ was measured by ELISPOT. Results represent the mean ± standard deviation (n=2). T cells primed with an irrelevant flu peptide were used as a negative control

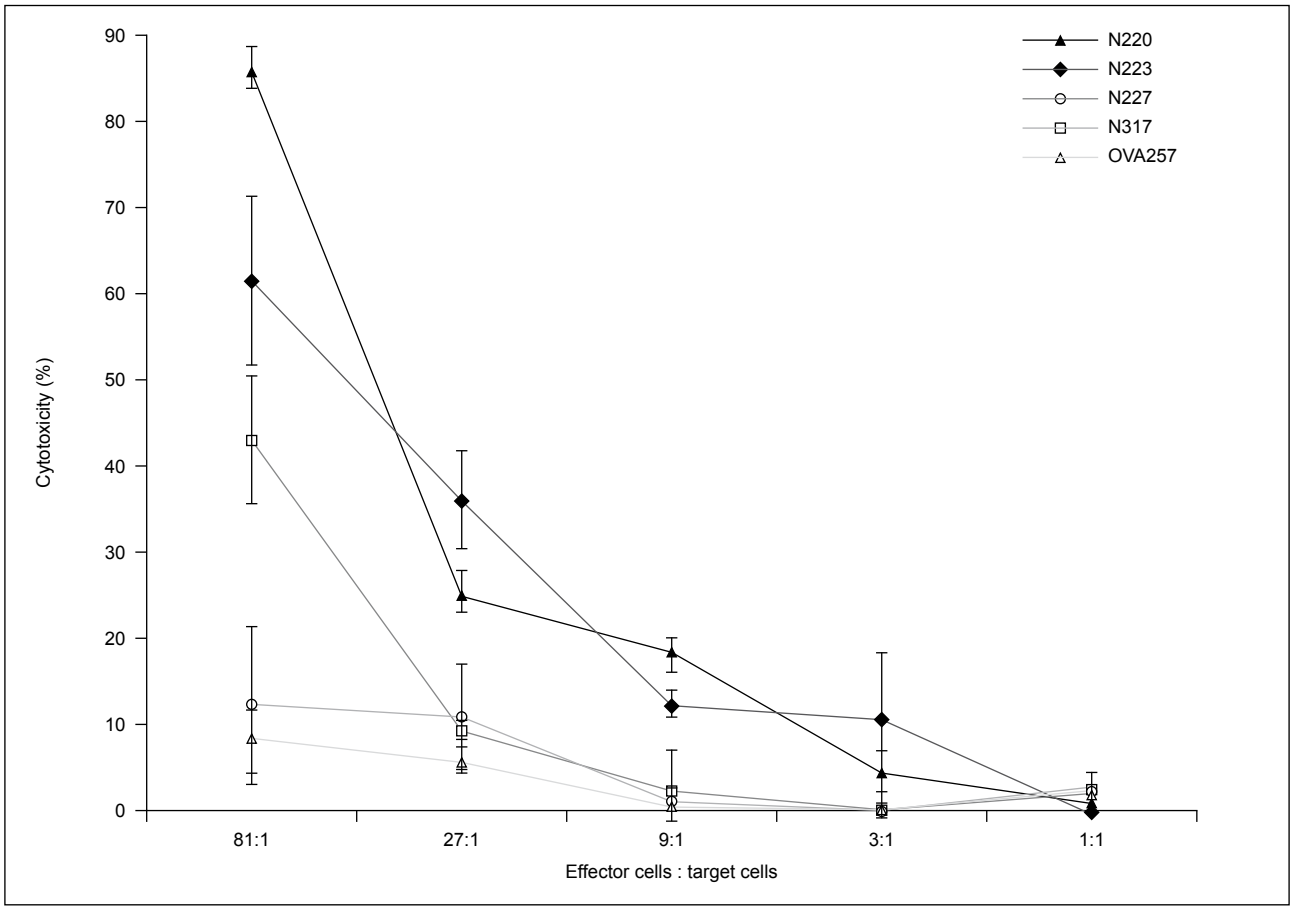

Fig 3. Cytotoxicity of spleen T cells against N/E6E7/A2.1K^b cells after DNA vaccination
Spleen T cells were harvested 1 week after the last vaccination and their cytotoxic activity against the N-protein expressing cells was compared based on LDH release. The X-axis indicates the different ratios of the effector cells (splenocytes) to the target cells (N/E6E7/A2.1K^b). The Y-axis indicates the cytotoxicity percentage. Four groups of mice were vaccinated with the N-protein peptide plasmids, N220MHKpVAX1 (indicated by -▲-); N223MHKpVAX1 (indicated by -◆-); N227MHKpVAX1 (indicated by -O-); and N317MHKpVAX1 (indicated by -□-). Mice vaccinated with an irrelevant plasmid, OVAMHKpVAX1 (indicated by -Δ-), were used as negative controls. The cytotoxicity was calculated as described in the materials and methods and the difference between the N220 peptide and the other peptides in the effector cells. A target cell ratio of 81:1 was calculated using the *t* test ($P<0.05$)

N223 peptide and significantly higher than that produced by the N227 and N317 peptides previously described,[4] results consistent with those of the T2-cell binding assay.

An SCT display DNA vaccine mechanism was then used to test the four most immunogenic peptides (N220, N223, N227 and N317) selected in the T2-cell binding assay and the human T-cell stimulation assay for their ability to trigger an immune response against SARS N-protein expressing cells in a transgenic mouse model expressing the human HLA-A2.1. In the SCT display system, the immunogenic peptide is translated together with the β_2-microglobulin and the MHC class I heavy chain molecule as a complex. After translation, the whole covalently linked MHC class I-peptide complex can be transferred from the endoplasmic reticulum to the cell surface for antigen presentation. The high stability of the covalently linked MHC class I-peptide complex produced from the SCT system excludes competing peptides and is thus a potent stimulant for T cells. Hence, it eliminates the uncertainty of antigen processing in the professional antigen presenting cells. The cytotoxic T cells can be primed more directly to ensure that the peptide encoded in the vaccine can be presented for vaccination. The cytotoxic T-cell response triggered by the DNA vaccine was investigated by measuring spleen T cells' ability to kill the N-protein expressing target cells, N/E6E7/A2.1K^b, 1 week after the last vaccination. The cytotoxicity level was measured by the amount of LDH released from the target cells. The results demonstrated that the DNA vaccines encoding the N-protein peptides N220 and N223 triggered the highest T-cell cytotoxicity towards the N-protein expressing cells with 86% and 61% cytotoxicity levels respectively using an effector cells/target cells ratio of 81:1. This compares favourably with the cytotoxicity level exhibited by the previously described peptide N317, which triggered only 42% cytotoxicity, and N227, which had results similar to the negative control using an irrelevant OVA257 peptide for vaccination (Fig 3).[4] Comparative results of cytotoxicity assays of the tested peptides indicated that the N220 peptide represents one of

the most potent amino acid sequences of the N-proteins able to trigger a cytotoxic T-cell response.

Interestingly, most reported immunogenic T-cell epitopes of N protein, including the N220 described in this study, are found between the amino acid residues 220 and 362. Therefore it would be interesting to investigate whether a vaccine composed of these ~140 amino acid peptides (N220 to N362) coupled with the known SARS B-cell epitopes previously described[5] would effectively trigger a protective immune response against the SARS-CoV in humans.

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