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# Identification of T-cell epitopes of SARS-coronavirus for development of peptide-based vaccines and cellular immunity assessment methods

## Key Messages

1. Subjects recovered from SARS-CoV infection retain memory of cellular immune response to epitopes spanning over a few regions of the viral nucleoprotein. Ten such epitopes were identified.
2. The majority of epitopes were predominantly recognised by CD8<sup>+</sup> cytotoxic T cells and elicited persistent memory response lasting at least 2 years.
3. There is potential to use the nucleoprotein of SARS-CoV to develop vaccines and diagnostic assays based on such cellular immune responses.

## Introduction

Severe acute respiratory syndrome (SARS) killed more than 800 people during a global outbreak in 2003. Specific antiviral treatment and prophylactic vaccine for SARS coronavirus (CoV) are not available. Among proteins encoded by the CoV family, the nucleoprotein is the most abundantly expressed and immunodominant. The nucleoprotein of SARS-CoV is crucial in the infection and pathogenic process, and is a highly sensitive diagnostic tool to detect SARS-CoV-specific antibody production.

Although the SARS-CoV nucleoprotein does not induce neutralising antibody, a nucleoprotein-specific cytotoxic T lymphocyte response is present in individuals who recovered from the disease. There is a potential to induce such an immune response by vaccination. This study examined the cytotoxic T lymphocyte epitopes of nucleoproteins from subjects who recovered from the SARS outbreak in Hong Kong in 2003.

## Methods

This study was conducted from 1 October 2005 to 30 September 2007. We recruited 55 subjects who had recovered from SARS-CoV infection and 10 controls without a history of SARS and tested negative for SARS-CoV-specific antibodies. A peripheral blood sample was collected 3, 10 to 12, and 22 to 24 months after the onset of illness for peripheral blood mononuclear cell (PBMC) extraction.

The interferon-gamma (IFN- $\gamma$ ) release enzyme-linked immunospot (ELISpot) assay was used to measure T-cell response to in vitro stimulation with peptides derived from the SARS-CoV nucleoprotein. The MultiScreen IP plate (Milipore, Bio-Gene) was coated with human IFN- $\gamma$  capture antibodies (R&D Systems). Cryo-preserved PBMCs were thawed and resuspended in culture medium RPMI 1640 containing 10% human AB serum (Sigma). A 100- $\mu$ L aliquot containing  $1.5 \times 10^5$  viable cells was seeded per well (in triplicate) and mixed with testing peptides at a final concentration of 10  $\mu$ g/mL. Concanavalin A (Sigma) at 0.5  $\mu$ g per well was used as a mitogen control. The positive peptide pool comprised 12 peptides derived from cytomegalovirus and Epstein-Barr virus. Cell controls were PBMCs alone without pulsing with any peptide. After an overnight incubation, the plate was washed. Human IFN- $\gamma$  detection antibody (R&D Systems) was added to each well and incubated overnight at 4°C. On the next day, the plate was washed, followed by the addition of streptavidin-alkaline phosphatase. After 2 hours of incubation at room temperature, the plate was washed again and followed by colour development using the Human IFN-gamma ELISpot development module (R&D Systems). The net number of IFN- $\gamma$ -producing cells for each peptide-test well was obtained by subtracting the mean spot-forming cell (SFC) counts of the corresponding triplicate cell control wells. A peptide or peptide pool was regarded as producing a positive response when the average net SFC count of the triplicate peptide test wells was greater than the mean plus two standard deviations (SD) of the cell controls, and having at least 20 SFCs.

*Hong Kong Med J* 2011;17(Suppl 6):S26-30

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RFCID project number: 03040602

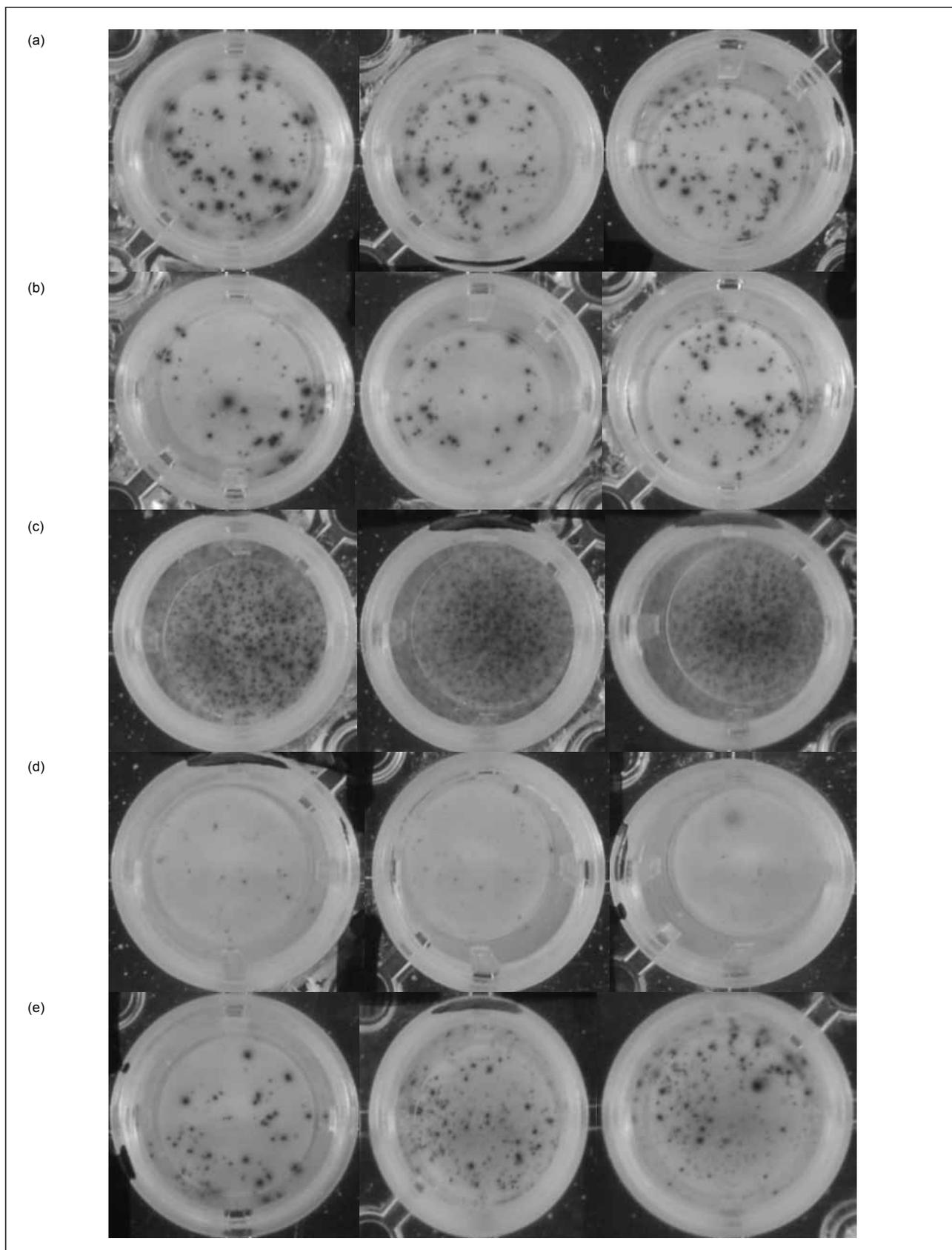
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**Fig 1. Typical examples of IFN- $\gamma$ -producing cells detected by ELISpot assay 3 months after onset of SARS**

Each IFN- $\gamma$ -producing cell appears as a dark spot with solid centre and faint border. (a) P-18 (containing peptide N48) and (b) P-21 (containing peptide N63) peptide pools show positive screening results and responses on fine mapping. (c) Peripheral blood mononuclear cells (PBMCs) stimulated with Concanavalin A, which is a mitogen to serve as a positive control to indicate cells, are viable and capable of producing IFN- $\gamma$ . (d) Cell controls are PBMCs not pulsed with any peptide. (e) Positive peptide pool (containing cytomegalovirus and Epstein-Barr virus T-cell epitopes) is recognised by HLA types commonly found in southern Chinese

**Table. Response to SARS-CoV nucleoprotein-derived peptides measured by IFN- $\gamma$  ELISpot assay in subjects who recovered from SARS**

Peptide name	% of subjects with a positive response in spot-forming cell counts			
	HLA-A*02 phenotype (n=28)	HLA-A*11 phenotype (n=25)	HLA-A*24 phenotype (n=13)	HLA-A*33 phenotype (n=8)
N15 (amino acid position 71-85)	0	8.0	15.4	0
N17 (amino acid position 81-95)	0	8.0	0	0
N18 (amino acid position 86-100)	10.7	24.0	92.3	0
N22 (amino acid position 106-120)	0	4.0	7.7	0
N25 (amino acid position 121-135)	3.6	0	0	0
N48 (amino acid position 236-250)	21.4	72.0	23.1	0
N53 (amino acid position 261-175)	3.6	12.0	0	37.5
N63 (amino acid position 311-325)	85.7	48.0	30.8	25.0
N68 (amino acid position 336-350)	28.6	20.0	0	75.0
N71 (amino acid position 351-365)	25.0	24.0	0	87.5

Peptides used were 15-mer overlap by 10 amino acids covering the whole nucleoprotein, except the last two amino acids at the C-terminus. The 3-month samples were screened with peptide pools. Subjects showing positive results were further tested with individual peptides. To determine the longevity of immune response memory, the 1-year and 2-year samples were tested in parallel against the reactive peptides. To further characterise the identified T-cell epitopes, PBMCs were pretreated to select CD8<sup>+</sup> cells and retested in parallel with the whole PBMC preparation.

## Results

Of the 55 subjects, 26 males and 26 females aged 21 to 48 (mean, 29; SD, 7.2) years had provided complete follow-up samples. The phenotype frequencies were as follows: A\*11 (61.5%), A\*02 (53.8%), A\*24 (17.3%), A\*33 (15.4%); A\*01, A\*03, and A\*30 (3.8% each); and A\*26, A\*29, A\*31 (1.9% each). The phenotype frequencies for DRB1 were as follows: DRB1-09 (40.4%), -12 (32.7%), -15 (21.2%), -04 (23.1%), -14 (17.3%), -11 (11.5%), -08 and -16 (7.7% each), -03 and -07 (5.8% each), and -10 (3.8%).

### Peptide pool screening

All subjects who recovered from SARS showed positive screening results to at least one peptide pool. The SFC counts for the peptide pools were 12 to 198 per  $1.5 \times 10^5$  PBMCs, whereas those for the cell controls were 0 to 12 per  $1.5 \times 10^5$  PBMCs. A typical example of ELISpot results is shown in Fig 1.

### Fine epitope mapping

The 3-month samples were further tested with individual peptides selected according to the screening results. Ten peptides were found to produce a positive ELISpot. The overall positive rates were: N63 (24 of 52 subjects, 46.2%), N48 (34.6%), N68 (25.0%), N18 (23.1%), N71 (21.2%), N53 (7.7%), N15 and N17 (3.8% each), N22 and N25 (1.9% each). The positive rates according to HLA phenotyping are shown in the Table. None of the controls showed positive ELISpots.

### Cross-sectional and longitudinal analyses on peptide response

The five peptides (N18, N48, N63, N68, and N71) that showed most frequent positive response were tested with the 1-year and 2-year PBMC samples to define the longevity of the T-cell response memory. Overall, the median SFC counts were 40 to 70 (range, 20-168) per  $1.5 \times 10^5$  PBMCs. To compare the response between peptides, the SFC counts for the five peptides obtained from PBMCs collected at 3 months post-illness onset were not significantly different ( $P=0.293$ , Kruskal-Wallis test). Similarly, the SFC counts for 1-year and 2-year time points were not significantly different ( $P=0.187$  for 1-year,  $P=0.434$  for 2-year, Fig 2). Similarly, the SFC counts for each peptide across the three specimen collection time points were not significantly different (Kruskal-Wallis test).

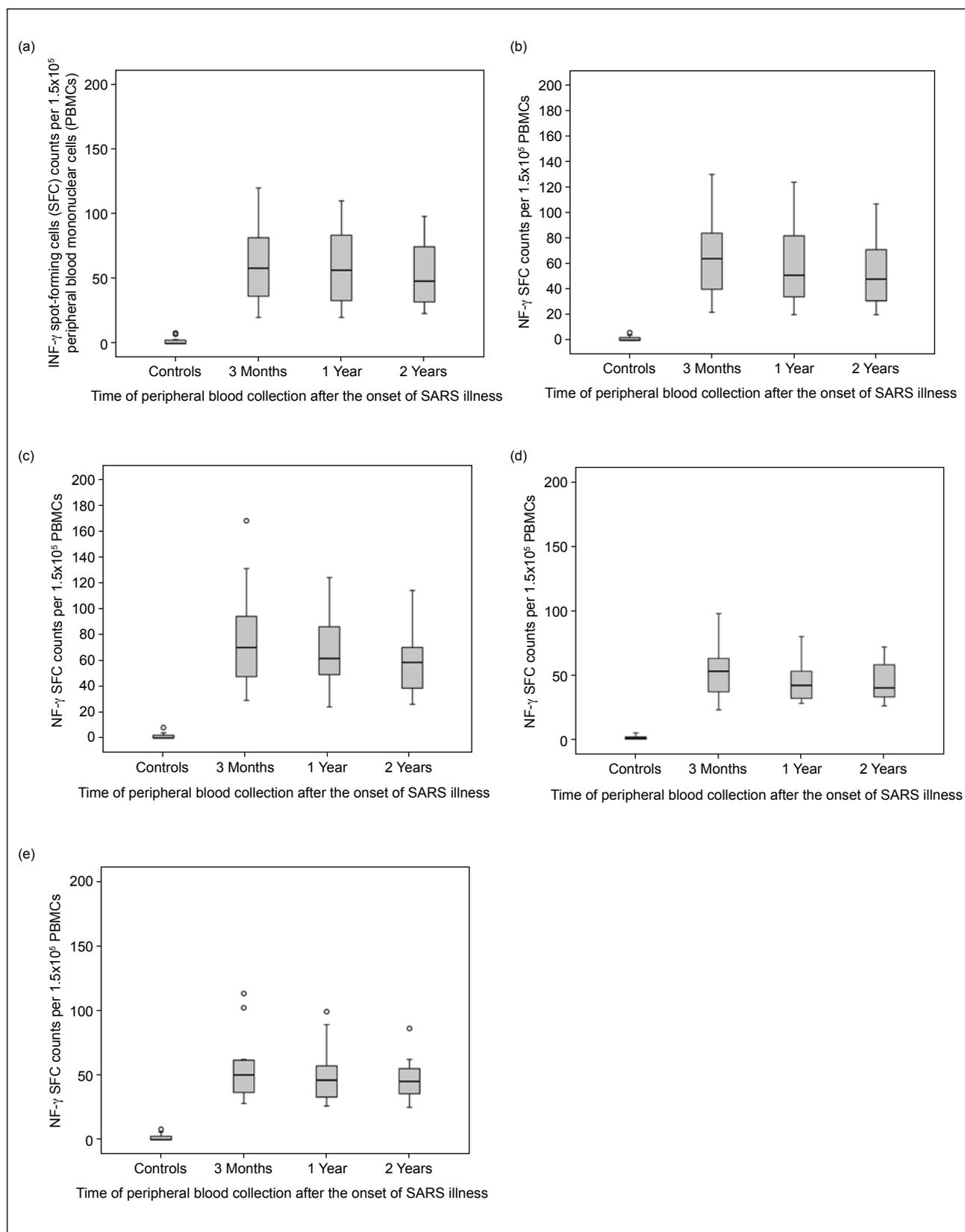
### CD8<sup>+</sup> cell enrichment

Five subjects showed a strong response to the corresponding peptide and had sufficient cells remaining for the CD8<sup>+</sup> cell enrichment study to characterise the five major epitopes identified. There was an increase in median SFC count for the CD8<sup>+</sup> cell-enriched preparation from 82 to 195 per  $1.5 \times 10^5$  cells for N18, from 76 to 210 for N48, from 102 to 315 for N63, from 66 to 155 for N68, and from 62 to 205 for N71.

## Discussion

Infection with SARS-CoV induced T-cell response memory targeting the nucleoprotein. There were 10 T-cell epitopes located within four regions (residues 71-100, 106-135, 236-175, and 336-365) of the nucleoprotein. These epitopes were mainly HLA type-specific where A\*02 recognised an epitope within residues 311-325 (N63), A\*11 within residues 236-250 (N48), A\*24 within residues 86-100 (N18), and A\*33 within residues 351-365 (N71).

A similar approach identified T-cell epitopes within residues 331-347, 339-354, and 346-362.<sup>1</sup> The residues 331-347 and 339-354 corresponded to the N68 peptide, in



**Fig 2. Frequency of  $INF-\gamma$ -producing cells in response to SARS-CoV nucleocapsid peptide stimulation according to the time of blood-sample collection\***

(a) N18 (amino acid position 86-100) for 12 subjects who recovered from SARS and 10 controls, (b) N48 (amino acid position 236-250) for 18 subjects who recovered from SARS and 10 controls, (c) N63 (amino acid position 311-325) for 24 subjects who recovered from SARS and 10 controls, (d) N68 (amino acid position 336-350) for 13 subjects who recovered from SARS and 10 controls, and (e) N71 (amino acid position 351-365) for 11 subjects who recovered from SARS and 10 controls

\* Circles represent outliers. None of the 10 non-SARS controls shows a positive response

line with the earlier study.<sup>1</sup> N68 elicited responses in A\*02, A\*11 and A\*33 subjects. The peptide N63 (residues 311-325) predominantly recognised by A\*02 subjects is able to induce recall memory from CD8<sup>+</sup> T cells.<sup>2</sup> The A\*33-associated peptide N71 (residues 351-265) is immunogenic in a mouse model.<sup>3</sup> Several helper T-cell epitopes based on a mouse model have been identified,<sup>3</sup> including one located within residues 111-125, which overlaps with our peptide N22 that was shown to elicit a positive response in 4.0% of A\*11 and 7.7% of A\*24 subjects.

An A\*02 epitope was predicted at residues 227-235 based on a T2 cell-peptide binding assay, but no significant response was observed when this peptide was tested with the PBMCs collected from four A\*02 subjects using the IFN- $\gamma$  release ELISpot assay.<sup>4</sup> We also did not observe a positive result towards this peptide—N46—from our tested subjects (including the 28 individuals with the A\*02 phenotype). Given the concurrent results from this and previous studies,<sup>4</sup> residues 227-235 are unlikely to represent a T-cell epitope.

Another A\*02 epitope was identified within residues 220-228 based on bioinformatics, and showed that a DNA vaccine encoding this epitope could induce cytotoxic immunity in a mouse model.<sup>5</sup> This potential peptide is within the N44 in our peptide pool, but no positive response was observed. Whether the spectrum of immune response between natural infection and vaccination differs remains to be defined. Our ELISpot results did not reveal a significant difference in SFC counts among samples taken 3 months, 1 year, and 2 years after the onset of illness. An immune

response memory was detected from PBMC samples taken 2 years after the onset of illness.<sup>1</sup> Thus, cellular immune response memory following SARS-CoV infection persists for a substantial period (at least 2 years). Although the humoral immune response memory was not measured, it is likely that it too persists. These are encouraging observations for the development of a vaccine for SARS-CoV infection.

### Acknowledgement

This study was supported by the Research Fund for the Control of Infectious Diseases (#03040602), Food and Health Bureau, Hong Kong SAR Government.

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