Memory T Cell Responses Targeting the SARS Coronavirus Persist up to 11 Years Post-Infection

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Abstract

Severe Acute Respiratory Syndrome (SARS) is a highly contagious infectious disease which first emerged in late 2002, caused by a then novel human coronavirus, SARS coronavirus (SARS-CoV). The virus is believed to have originated from bats and transmitted to human through intermediate animals such as civet cats. The re-emergence of SARS-CoV remains a valid concern due to the continual persistence of zoonotic SARS-CoVs and SARS-like CoVs (SL-CoVs) in bat reservoirs. In this study, the screening for the presence of SARS-specific T cells in a cohort of three SARS-recovered individuals at 9 and 11 years post-infection was carried out, and all memory T cell responses detected target the SARS-CoV structural proteins. Two $CD8^+$ T cell responses targeting the SARS-CoV membrane (M) and nucleocapsid (N) proteins were characterized by determining their HLA restriction and minimal T cell epitope regions. Furthermore, these responses were found to persist up to 11 years post-infection. An absence of cross-reactivity of these $CD8^+$ T cell responses against the newly-emerged Middle East Respiratory Syndrome coronavirus (MERS-CoV) was also demonstrated. The knowledge of the persistence of SARS-specific celullar immunity targeting the viral structural proteins in SARS-recovered individuals is important in the design and development of SARS vaccines, which are currently unavailable.

Keywords

SARS-CoV

T cell

Immunity

Epitope

Abbreviations

SARS, severe acute respiratory syndrome

SARS-CoV, SARS coronavirus

1. Introduction

Severe Acute Respiratory Syndrome (SARS) first emerged 12 years ago as a highly contagious infectious disease, caused by a then novel human coronavirus, termed SARS coronavirus (SARS-CoV) [1]. The virus spread to 25 countries in a short period of three months, affecting a total of 8098 people globally including 774 deaths, a fatality rate of 10% [2]. SARS-CoV is believed to have originated from bats [3-5] and transmitted to human through intermediate animals such as civet cats [6]. Although no SARS cases have been reported since 2004, the re-emergence of SARS-CoV is of public health concern due to the continual persistence of SARS-CoVs and SARS-like CoVs (SL-CoVs) in bat reservoirs. The SARS-CoV is classified in the order *Nidovirales*, family *Coronaviridae* and genus betacoronavirus (lineage B). It is an enveloped, positive-sense and single-stranded RNA virus of a genome size of 29.7 kb, encoding 16 non-structural proteins (nsps), 4 structural proteins (spike [S], membrane [M], nucleocapsid [N], envelope [E]) and 8 accessory proteins (3a, 3b, 6, 7a, 7b, 8a, 8b, 9) proteins [7].

Animal studies have indicated the importance of T cells in the clearance of SARS-CoV during primary infection and protection from disease [8-10]. In humans, decreased T cell numbers (lymphopenia) correlated with severe disease, indicating the critical role of T cell-mediated immune response in disease development [11, 12]. While SARS-specific antibody level in SARS-recovered individuals is undetectable at 6 years post-infection, SARS-specific memory T cells persisted up to 6 years following recovery [13]. The long-term persistence of memory T cell immunity could be important in protection against SARS-CoV re-infection. In this study, the presence of SARS-specific T cells was screened in three SARS-recovered individuals at 9 and 11 years post-infection. The characterization of $CD8^+$ T cell responses against the structural M and N proteins was carried out, including the determination of HLA restriction and the minimal T cell epitope. In addition, cross-reactivity of SARS-specific $CD8^+$ T cells against the Middle East Respiratory Syndrome coronavirus (MERS-CoV) was investigated.

2. Materials and methods

2.1. Synthetic peptides

A total of 550 peptides were purchased from Chiron Mimotopes (Victoria, Australia) at purity above 80% and their compositions were confirmed by mass spectrometry. The peptides are 15-mers overlapping by 10 residues spanning the proteome of SARS-CoV structural (S, E, M, N) and accessory (3a, 3b, 6, 7a, 7b, 8a, 8b, 9) proteins. Peptides were received in lyophilized forms and diluted at 40 mg/ml in dimethyl sulfoxide (DMSO) and then further diluted in RPMI medium (Gibco®) at working concentrations of 10 mg/ml to 1 mg/ml.

2.2. Collection of blood samples from SARS-recovered subjects

Three SARS-recovered individuals were enrolled from the Singapore General Hospital, Singapore. All participants were diagnosed with SARS during the period of 2003 according to World Health Organization's definition of SARS [14]. Blood samples were obtained at either 9 or 11 years post-infection. This study was approved by the Singhealth Centralized Institutional Review Board (Singapore).

2.3. PBMC isolation and *in vitro* expansion of SARS-specific T cells

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh heparinized blood by density gradient centrifugation using Ficoll-Paque^{TM} (GE Life Sciences) and resuspended in AIM-V medium (Invitrogen) with 2% pooled human AB serum (AIM-V+2%AB). Cells were either frozen down or used directly for *in vitro* expansion in the presence of SARS peptides, as previously described [13].

2.4. Anti-human IFNγ ELISpot assay

Anti-human IFNγ enzyme-linked immunospot (ELISpot) assays were performed as previously described [13], using the SARS peptides arranged in numeric and alphabetic matrix pools

(Supplementary Table 1). The positive threshold was set at number of spot-forming units (SFU) per 5 x 10^4 cells at least twice of that observed in negative control (cells not stimulated with peptides). The peptide responsible for positive ELISpot results was identified as the common peptide present in both the numeric and alphabetic pools.

2.5. Intracellular cytokine staining (ICS) and degranulation assays

In vitro expanded PBMCs were incubated in AIM-V+2%AB medium alone or with peptides at 5 μg/ml for 5 hours in the presence of 10 μg/ml of brefeldin A. Anti-CD107a-FITC antibody (BD Pharmingen) was added for assessing $CD8^+$ T cell degranulation. Positive control consisted of T cells incubated in AIM-V+2%AB with 10 ng/ml phorbol 12-myristate 13-acetate (PMA) and 100 ng/ml ionomycin. Following stimulation, cells were washed in Hank's Balanced Salt Solution (HBSS [Gibco®]) and stained with anti-CD8-phycoerythrin(PE)-Cy7 and anti-CD3-peridinin chlorophyll protein(PerCP)-Cy5.5 (BD Pharmingen) at 4^oC. Cells were washed in 1x phosphate buffered saline (PBS) containing 1% BSA and 0.1% azide, fixed and permeabilized using Cytofix/Cytoperm fixation/permeabilization reagent (BD Biosciences) according to manufacturer's protocol. Intracellular staining using anti-IFNγ-PE (BD Pharmingen) was carried out at 4^oC, followed by washing and flow cytometry analysis.

2.6. Human Leukocyte Antigen (HLA) restriction of $CD8^+$ T cell responses

HLA class I phenotypes of the SARS-recovered subjects was determined by PCR amplification and sequencing-based typing method as previously described [13] and as performed by BGI Clinical laboratories (ShenZhen, China). Epstein-Barr virus-transformed lymphoblastoid B cell lines (EBV-LCLs) possessing matching HLA phenotypes as the subjects were used as antigen-presenting cells (APCs) to determine the HLA restriction of $CD8^+$ T cell responses.

2.7. Restimulation of SARS-specific $CD8^+$ T cells and minimal epitope mapping of $CD8^+$ T cell epitopes

Restimulation of SARS-specific $CD8^+$ T cells was done using fresh PBMCs from a healthy donor and EBV-LCL consisting of the HLA allele restricting the $CD8^+$ T cell response. Specific peptide was added to EBV-LCL at 1 μg/ml in R10 medium and incubated at 37 ^oC for 1 hour, followed by washes with HBSS. PBMCs and the peptide-pulsed EBV-LCL were irradiated at 2500 RADs and 4000 RADs respectively, washed with HBSS and added to *in vitro* expanded T cells in AIM-V+2%AB supplemented with IL-2 (20 U/ml), IL-7 (10 ng/ml) and IL-15 (10 ng/ml) and co-cultured at 37^oC for 10 days.

For mapping of minimal T cell epitope, restimulated short-term T cell lines were tested with truncated peptides of the 15-mer peptide by ICS. For M29 minimal epitope mapping, 21 peptides (8-mers to 12-mers) spanning the M29 region were tested. For N53 minimal epitope mapping, 6 peptides (8-mers to 10-mers) spanning the overlapping region of N53 and N54 were used.

3. Results and Discussion

3.1. Identification of SARS-specific memory T cell responses in SARS-recovered individuals at 9 and 11 years post-infection

As it is currently unknown if SARS-specific memory T cell responses persist in SARS-recovered individuals after 6 years post-infection, PBMCs from a SARS convalescent subject (SARS subject 1) were collected at 9 years post-infection and tested for SARS-specific memory T cells. As negative control, PBMCs of a healthy individual with no SARS history were also obtained and tested. After *in vitro* expansion with the mixture of SARS 15-mer peptides of 10 overlapping residues spanning the structural (S, E, M, N) and accessory (3a, 3b, 6, 7a, 7b, 8a, 8b, 9b) proteins, the PBMCs were subjected to IFNγ ELISpot assay using SARS peptide pools arranged in alphabetic and numeric matices (Supplementary Table 1). Analysis of ELISpot results was performed with the positive threshold set as the number of spot-forming units (SFU) two times above the mean SFU of unstimulated cells. As shown in Figure 1, higher frequencies of IFNγ-producing SFUs were observed for *in vitro*-expanded PBMCs from SARS subject 1 compared to the healthy individual, suggesting

the presence of SARS-specific memory T cells at 9 years post-infection. These responses were low in frequency since *in vitro* expansion of PBMCs was required for their detection. This is in agreement with previous reports that reported the decline of memory T cell responses in SARS convalescent individuals over time [13, 15].

Peptides inducing IFNγ production as identified from ELISpot were further tested by ICS to confirm their abilities to elicit specific T cell IFNγ response and to define the subset of T cells ($CD4^+$ or $CD8^+$) involved. A total of 4 SARS-specific memory T cell responses were identified in SARS subject 1 and they are specific for structural S, N and M proteins (Table 1). Three are $CD4^+$ T cell responses, of which two recognized the S protein (S104 and S109) and one recognized the N protein (N21). In addition, $CD8^+$ memory T cell response specific for the SARS-CoV M protein (M29) was detected. Subsequently, PBMCs were obtained from two other SARS-recovered individuals (SARS subject 2 and 3) at 9 and 11 years post-infection respectively and screened for SARS-specific memory T cells using the same method. Memory T cell responses specific against SARS-CoV structural proteins were also found (Table 1). As with that observed in SARS subject 1, N21 $CD4^+$ response and M29 $CD8^+$ response were found in SARS subject 2 and 3 respectively. SARS subject 3 also possessed a $CD4^+$ T cell response targeting S217. As summarized in Table 1, subject 1 had more SARS-specific memory T cells at higher frequencies compared to the other two subjects. It was noted that subject 1 had more severe disease presentation (Supplementary Table 2), which could be related to the more robust T cell responses detected. However, the number of subjects recruited in this study is too small to draw a conclusion to this correlation.The knowledge that SARS-CoV structural proteins are highly immunogenic in eliciting protective and immunodominant T cell responses is well-established [16-18]. The $CD4^+$ T cell epitopes identified here, which are specific against S104 (S protein residues 516-530), S109 (S protein residues 541-555), S217 (S protein residues 1081-1095) and N21 (N protein residues 101-115), have

been previously reported from a cohort of SARS-recovered patients at 1 year post-infection, suggesting the immunoprevalence and dominance of these responses in convalescent SARS patients [16]. Here, the identification of T cell responses against SARS-CoV structural S, N and M proteins at 9 and 11 years post-infection suggests the long-term persistence of these responses.

3.2. Characterization of SARS-specific M29 $CD8^+$ T cell response

The $CD8^+$ T cell response present in SARS subject 1 and 3, which is specific for SARS peptide M29 corresponding to residues 141-155 of the structural M protein, was further characterized. Using T cells from subject 1, the M29 $CD8^+$ T cell response was determined to be restricted by the HLA-B*1502 allele. As revealed by ICS, M29-restimulated $CD8^+$ T cells exhibited $CD8^+IFN\gamma^+$ response at 27.6% when stimulated with M29 peptide (Figure 2, left panels). Additionally, CD107a expression of T cells induced by M29 peptide was determined to be 12.7% (Figure 2, right panels). The increase in CD107a expression, a marker for T cell degranulation and target cell-killing function via the perforin-granzyme pathway [19], indicates that the memory T cells were capable of degranulation and likely to exhibit target cell-killing function upon activation by M29 peptide.

HLA class I molecules preferentially bind and present peptides of 8 to 11 amino acids to be recognized by HLA receptors on $CD8^+$ T cells during T cell activation [20]. Since the M29 peptide is a 15-mer peptide, the identification of the position and minimal number of amino acids within the M29 region, known as the minimal epitope, capable in eliciting the M29 $CD8^+$ T cell response was carried out. To do so, truncated peptides within the M29 region ranging from 8- to 12-mers were tested for their abilities to induce IFNγ secretion by M29-restimulated T cells. As shown in Table 2, the 9-mer peptide, $M29_{147\text{-}155}$, corresponding to residues 147-155 of M protein, was most efficient in inducing the $CD8^+$ T cell response, resulting in the highest percentage of IFNγ-producing cells of 32.9%. This 9-mer also represents the minimal epitope of M29 $CD8^+$ T cell response, as the removal of either the N-terminus histidine (H) residue ($M29_{148\text{-}155}$) or the C-terminus leucine (L) residue ($M29_{145\text{-}154}$) completely abolished IFNγ production (Table 2).

In a study involving 128 SARS convalescent patients at 1 year post-infection, $CD8^+$ T cell response against residues 146-160 of the M protein was present in 9% of study subjects, but the minimal epitope and the HLA-restriction of this response were not determined [16]. The M29 minimal epitope (residues 147-155) identified in present study lies within this reported region. Other T cell epitopes, both $CD4^+$ and $CD8^+$, within the SARS-CoV M protein have also been reported [17, 21]. In another study looking at SARS-specific memory T cell responses in SARS-recovered individuals at 4 years post-infection, 28.75% of them presented T cell responses to M peptides [22], further supporting the role of M protein in eliciting dominant cellular immunity during SARS-CoV infection.

3.3. Characterization of SARS-specific N53 $CD8^+$ T cell response

The SARS-CoV N protein is capable in inducing immunodominant T cell responses in SARS-recovered individuals and these responses were shown to be involved in disease protection in animal models [23, 24]. In our previous study performed at 6 years post-SARS, several SARS-specific T cell epitopes within the N protein were reported [13]. In SARS subject 1 at 6 years post-infection, a HLA-B*1525-restricted memory $CD8^+$ T cell response targeting the N53 peptide, corresponding to residues 261-275 of N protein, was detected. To determine the minimal epitope of the N53 $CD8^+$ T cell response, truncated peptides were tested for induction of $CD8^+$ T cell response using PBMCs from SARS subject 1 previously collected at 6 years post-infection. Truncated peptides consisted of 8- to 10-mers within the 10 overlapping residues between N53 and N54 peptides, as the N54 peptide is also capable of inducing the response (data not shown). It was found that 10-mer peptide, $N53_{266\text{-}275}$, corresponding to residues 266-275 of the N protein, was most efficient in inducing N53 T cell response of 12.7% (Table 3). Deletion of N-terminal threonine (T) residue and C-terminal phenylalanine (F) residue from $N53_{266\text{-}275}$ led to decrease in percentages of IFNγ-producing $CD8^+$ T cells to 10.9% and 8.0% respectively. This indicates that residues 266-275 is the minimal epitope of the N53 $CD8^+$ T cell response. In previous study using bioinformatics NetMHCpan algorithm, the predicted minimal epitope for the N53 response was determined to be 9 amino acids at position 267-

275 [25], which is within the 10-mer region identified in current study. Thus far, no other studies have reported the identification of the N53 $CD8^+$ T cell epitope.

3.4. Persistence of memory SARS-specific M29 and N53 $CD8^+$ T cell responses at 11 years post-infection

Having characterized two $CD8^+$ T cell responses at 6 and 9 years post-infection, the same donor was recalled at 11 years post-infection to determine the persistence of these responses. PBMCs collected from the same individual were expanded *in vitro* using M29 and N53 minimal peptides ($M29_{147-155}$ and $N53_{266-275}$) and tested for $CD8^+IFN\gamma^+$ responses. As shown in Figure 3 (left panel), when cells were stimulated with $M29_{147-155}$ and $N53_{266-275}$ peptides, $CD8^+IFN\gamma^+$ T cell responses of 0.4% and 0.9% were observed respectively, suggesting the persistence of these SARS-specific memory T cells up to 11 year after infection. CD107a expression at 0.6% and 1.3% were also observed when cells were stimulated with $M29_{147-155}$ and $N53_{266-275}$ peptides respectively (Figure 3, right panels), indicating degranulation of T cells upon peptide stimulation. $CD4^+$ T cell responses detected in SARS subject 1 at 9 years post-infection (Table 1) were undetectable at 11 years post-infection (data not shown).

3.5. Lack of cross-reactivity of SARS-specific M29 and N53 $CD8^+$ T cell responses against MERS-CoV

A novel human coronavirus, MERS-CoV, first emerged in 2012 [26, 27]. Like SARS-CoV, MERS-CoV is a betacoronavirus which causes serious and sometimes fatal lower respiratory tract infections and extrapulmonary manifestations [28, 29]. Contrary to SARS-CoV which is a lineage B betacoronavirus, MERS-CoV belongs to lineage C [30]. To investigate if SARS-specific M29 and N53 $CD8^+$ T cells can cross-react with M and N peptides of MERS-CoV, sequence alignments were done to identify corresponding M29 and N53 minimal epitopes of MERS-CoV (Figure 4A and 4B). When M29- and N53-restimulated T cells were stimulated with MERS-CoV M29 minimal epitope peptide (HLKMAGMHF) and N53 minimal epitope peptide (TKSFNMVQAF), no $CD8^+IFN\gamma^+$

responses were observed (Figure 4C), indicating the inability of these SARS-specific T cells to be activated by MERS-CoV peptides. Therefore, T cell immunity against SARS-CoV is highly specific and M29 and N53 $CD8^+$ T cell responses are unlikely to provide cross-protection against MERS-CoV infection. This is expected as MERS-CoV is distantly related to SARS-CoV and is more closely related to other bat coronaviruses [30]. Nonetheless, sequence alignments revealed that the M29 and N53 minimal epitopes are fully conserved between human and zoonotic strains (Figure 4A and 4B), including civet SARS-CoV SZ3, bat SL-CoVs Rp3 and Rf1, and the bat SARS-CoV Rs3367 which is capable of utilizing both human and bat ACE2 receptors for cell entry [5]. Hence, it is likely that the SARS-specific M29 and N53 $CD8^+$ T cells can confer cross-protection against infections of these zoonotic SARS-CoV and SL-CoV strains.

4. Conclusion

There are currently no reports on the persistence of memory T cells in SARS-recovered individuals beyond 6 years post-infection, therefore, the longevity of SARS-CoV cellular immunity is unclear. In this study, it was demonstrated that SARS-specific memory T cells persist in three SARS-recovered individuals at 9 and 11 years post-SARS in the absence of antigen. All memory T cells detected were specific against SARS-CoV structural S, N and M proteins. Two immunodominant $CD8^+$ T cell responses specific against M (M29) and N (N53) proteins were further characterized by defining the minimal epitope and HLA restriction. These $CD8^+$ T cell responses continued to persist in a SARS-recovered subject up to 11 years post-infection. The persistence of T cell responses suggests that SARS-recovered patients could be protected from re-infection.

Peptides of the replicase protein, which comprises 2/3 of the SARS-CoV proteome, were not included in memory T cell screening in the current study due to limited amount of SARS subject PBMCs obtained. However, based on current literature, the SARS-CoV replicase protein is less immunogenic compared to structural proteins [16]. It was also noted that the availability of three convalescent individuals is a significant constraint of this study. In future studies, the conclusions drawn here could be substantiated by including more SARS-recovered subjects.

In a Phase I clinical trial involving a SARS DNA vaccine encoding the S protein, SARS-specific T cell responses were observed in vaccinated individuals, suggesting that the S protein is sufficiently to induce T cell responses [31]. In line with this, results in current study showed the long-term persistence of T cell responses targeting the S protein, as well as other structural proteins including M and N proteins. This provides evidence for the design of SARS vaccines comprising of the viral structural proteins for the induction of dominant, potent and long-lived memory cellular responses against the virus.

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Figure captions

Figure 1. IFNγ ELISpot results for SARS-specific memory T cell screening. PBMCs from (A) a healthy individual and (B) SARS-recovered individual (SARS subject 1) were expanded *in vitro* using a mixture of SARS-CoV peptides, followed by IFNγ ELISpot assay using SARS peptide matrix pools of the structural (top panels) and accessory proteins (lower panels). Each bar represents the IFNγ-producing response to an individual peptide matrix pool (numeric or alphabetic) in SFU per 5 x 10^4 cells. The threshold for a positive response was set as two times above the mean SFU of unstimulated

cells (Neg), as indicated by the dotted line in the right panels. Cells stimulated with PMA/ionomycin were included as positive control (Pos).

Figure 2. ICS and flow cytometry analysis of unstimulated and M29-stimulated T cells after restimulation using M29 peptide. The percentages of $CD8^+$ $IFN\gamma^+$ and $CD8^+CD107a^+$ T cells shown represent the percentage of the T cells in total T cell population (after gating the $CD3^+$ cells) present in the short-term T cell line obtained by restimulation using M29 peptide from SARS subject 1 at 9 years post-infection.

Figure 3. ICS and flow cytometry analysis of restimulated T cells from SARS subject 1 at 11 years post-infection. Percentages of $CD8^+IFN\gamma^+$ responses (left panels) and $CD8^+CD107a^+$ responses (right panels) of (A) unstimulated, (B) $M29_{147\text{-}155}$-stimulated, (C) $N53_{266\text{-}275}$-stimulated T cells are as indicated in the upper right quadrant of each dot plot. Percentage $CD8^+$ $IFN\gamma^+$ cells shown represent the percentage of IFNγ-producing cells in the total T cell population (after gating the $CD3^+$ cells) which were *in vitro* expanded in the presence of $M29_{147\text{-}155}$ and $N53_{266\text{-}275}$ peptides.

Figure 4. Cross-reactivity of SARS-specific M29 and N53 $CD8^+$ T cells. Sequence alignments of (A) M29 and (B) N53 regions of human SARS-CoV (HKU39849), civet SARS-CoV (SZ3), bat SL-CoVs (Rp3, Rf1 and Rs3367) and MERS-CoV. (C) Percentages of $CD8^+IFN\gamma^+$ T cell responses induced by SARS-CoV and MERS-CoV M29 (left) and N53 (right) minimal peptides. Percentage $CD8^+$ $IFN\gamma^+$ cells shown represents the percentage of IFNγ-producing cells in the total T cell population (after gating the $CD3^+$ cells) present in the short-term T cell line obtained by restimulation using SARS-CoV M29 and N53 minimal peptides ($M29_{147\text{-}155}$ and $N53_{266\text{-}275}$) from SARS subject 1 at 9 years post-infection.

401 **Tables**

	HLA Class I		Years post-SARS infection	Peptide	Amino acid position	Type of T cell response	Percentages of T cell responses after *in vitro* expansion
SARS subject 1	A*2402 B*1502 C*0801	A*0206 B*1525 C*0403	9 years	S104 S109 N21 M29	516 - 530 541 - 555 101 -115 141 - 155	$CD4^+$ $CD4^+$ $CD4^+$ $CD8^+$	3.9% 3.1% 4.7% 1.0%
SARS subject 2	A*1101 B*5502 C*0302	A*3303 B*5801 C*0303	9 years	N21	101 -115	$CD4^+$	0.2%
SARS subject 3	A*0201 B*1502 C*0801	A*1101 B*4001 C*1502	11 years	S217 M29	1081 - 1095 141 - 155	$CD4^+$ $CD8^+$	0.3% 0.3%

402 **Table 1. Summary of T cell responses in SARS-recovered subjects at 9 or 11 years post-infection,**
403 **identified from screening by ELISpot and confirmation by ICS.** Percentages of T cell responses
404 represent that of $CD4^+$ or $CD8^+$ T cells over total T cell population after *in vitro* expansion in the
405 presence of SARS peptide mixtures.

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Peptide	Peptide Length	Amino acid position	Peptide Sequence	Percentage of $CD8^+IFN\gamma^+$ T cells
M29	15-mer	141 - 155	AVIIR G***HLRM AGHSL***	5.3%
$M29_{144-155}$	12-mer	144 - 155	IR G***HLRM AGHSL***	14.8%
$M29_{143-154}$	12-mer	143 - 154	IIR GHLRM AGHS	0.2%
$M29_{145-155}$	11-mer	145 - 155	R G***HLRM AGHSL***	17.8%
$M29_{146-155}$	10-mer	146 -155	G***HLRM AGHSL***	22.8%
$M29_{145-154}$	10-mer	145 - 154	R GHLRM AGHS	0.2%
$M29_{147-155}$	9-mer	147 -155	***HLRM AGHSL***	32.9%
$M29_{148-155}$	8-mer	148 - 155	LRM AGHSL	0.3%
		Unstimulated cells		0.2%
		PMA/Ionomycin-stimulated cells		11.1%

Table 2. Summary of percentage $CD8^+$ $IFN\gamma^+$ responses in SARS subject 1 induced by truncated peptides within M29 region. T cells used were obtained from SARS subject 1 at 9 years post-infection. Results of positive peptides (M29, $M29_{144-155}$, $M29_{145-155}$, $M29_{146-155}$, $M29_{147-155}$) and selected negative peptides ($M29_{143-154}$, $M29_{145-154}$, $M29_{148-155}$) are shown. Percentage $CD8^+IFN\gamma^+$ T cells shown represents the percentage of IFNγ-producing $CD8^+$ T cells in the total T cell population (after gating the $CD3^+$ cells) present in the short-term T cell line obtained by restimulation using M29 peptide. The minimal epitope is indicated in italics.

Peptide	Peptide Length	Amino acid position	Peptide Sequence	Percentage of $CD8^+IFN\gamma^+$ T cells
N53	15-mer	261 - 275	QKRTA ***TKQYN VTQAF***	8.8%
$N53_{266-275}$	10-mer	266 - 275	***TKQYN VTQAF***	12.7%
$N53_{266-274}$	9-mer	266 - 274	TKQYN VTQA	8.0%
$N53_{267-275}$	9-mer	267 -275	KQYN VTQAF	10.9%
$N53_{266-273}$	8-mer	266 - 273	TKQYN VTQ	2.2%
$N53_{267-274}$	8-mer	267 - 274	KQYN VTQA	2.3%
$N53_{268-275}$	8-mer	268 - 275	QYN VTQAF	2.2%
		Unstimulated cells		1.6%
		PMA/Ionomycin-stimulated cells		15.3%

Table 3. Summary of percentage $CD8^+IFN\gamma^+$ responses in SARS subject 1 induced by truncated peptides within N53 region. T cells used were obtained from SARS subject 1 at 6 years post-infection. Percentage of $CD8^+IFN\gamma^+$ cells shown represent the percentage of IFNγ-producing $CD8^+$ cells in the total T cell population (after gating the $CD3^+$ cells) present in the short-term T cell line obtained from restimulation using N53 peptide. The minimal epitope is indicated in italics.

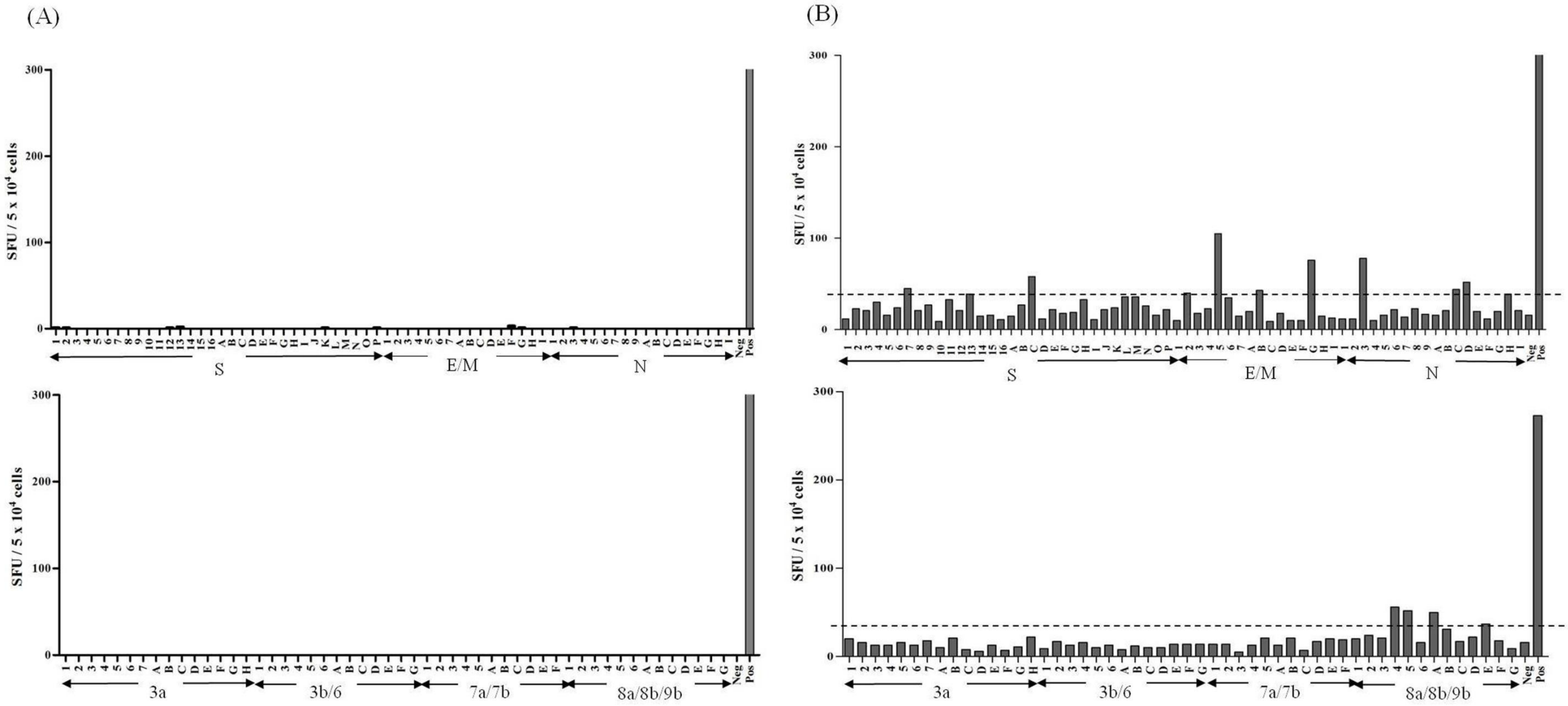
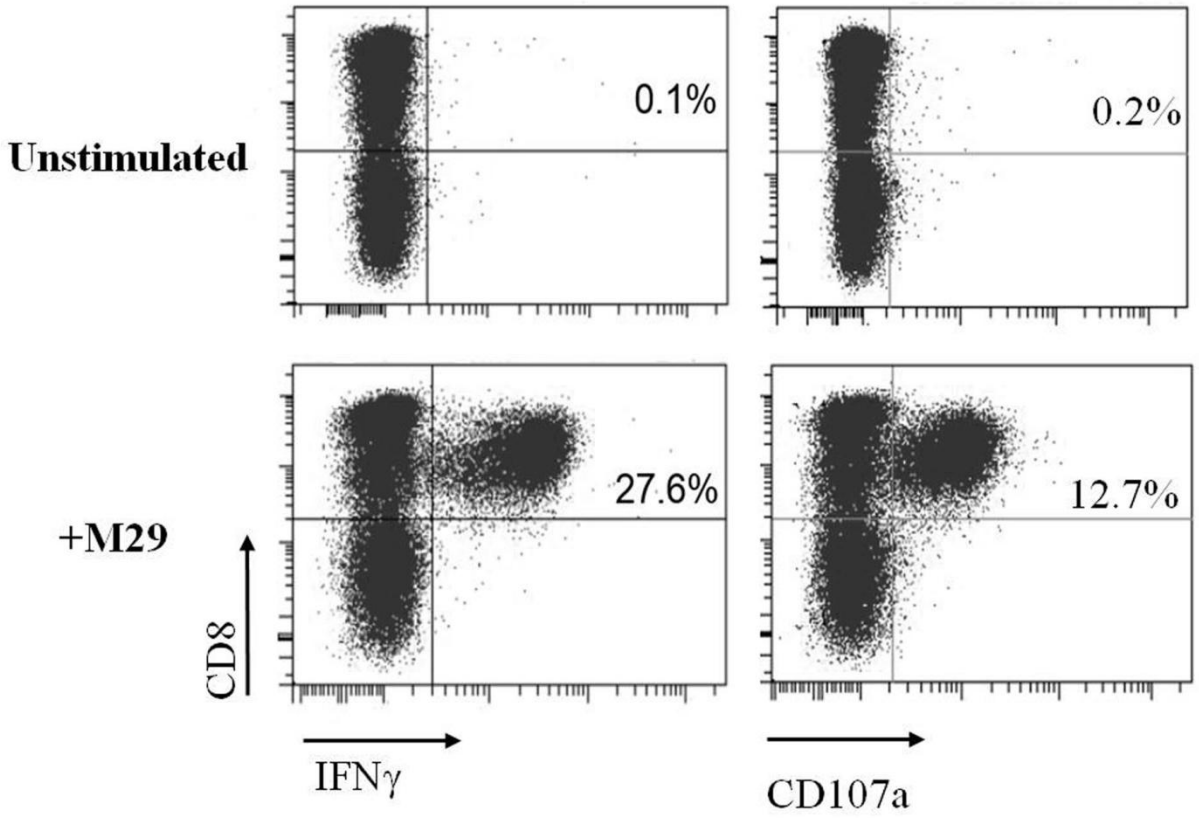


Figure 1. IFNγ ELISpot results for SARS-specific memory T cell screening. PBMCs from (A) a healthy individual and (B) SARS-recovered individual (SARS subject 1) were expanded *in vitro* using a mixture of SARS-CoV peptides, followed by IFNγ ELISpot assay using SARS peptide matrix pools of the structural (top panels) and accessory proteins (lower panels). Each bar represents the IFNγ-producing response to an individual peptide matrix pool (numeric or alphabetic) in SFU per 5 x 10^4 cells. The threshold for a positive response was set as two times above the mean SFU of unstimulated cells (Neg), as indicated by the dotted line in the right panels. Cells stimulated with PMA/ionomycin were included as positive control (Pos).



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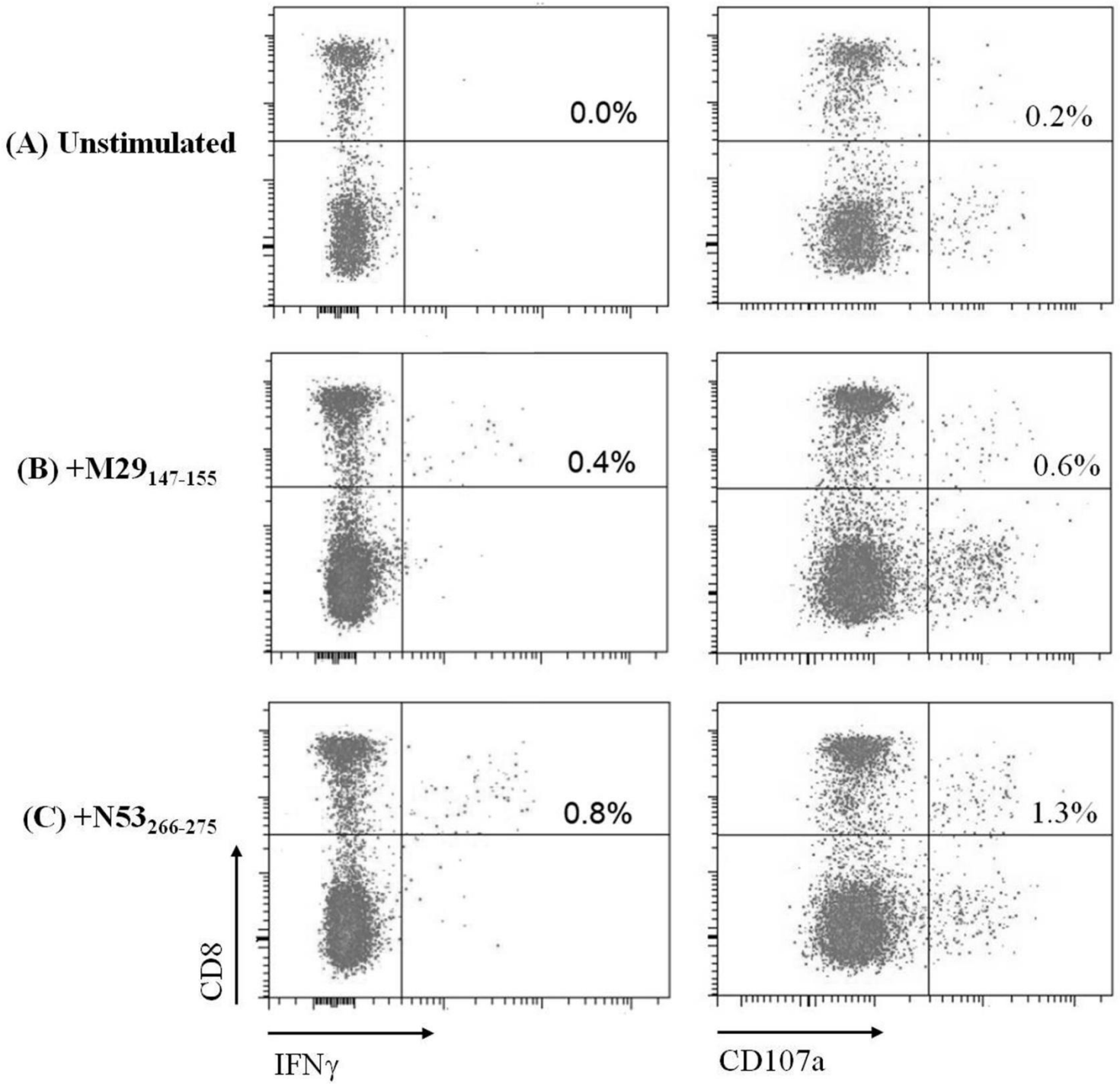

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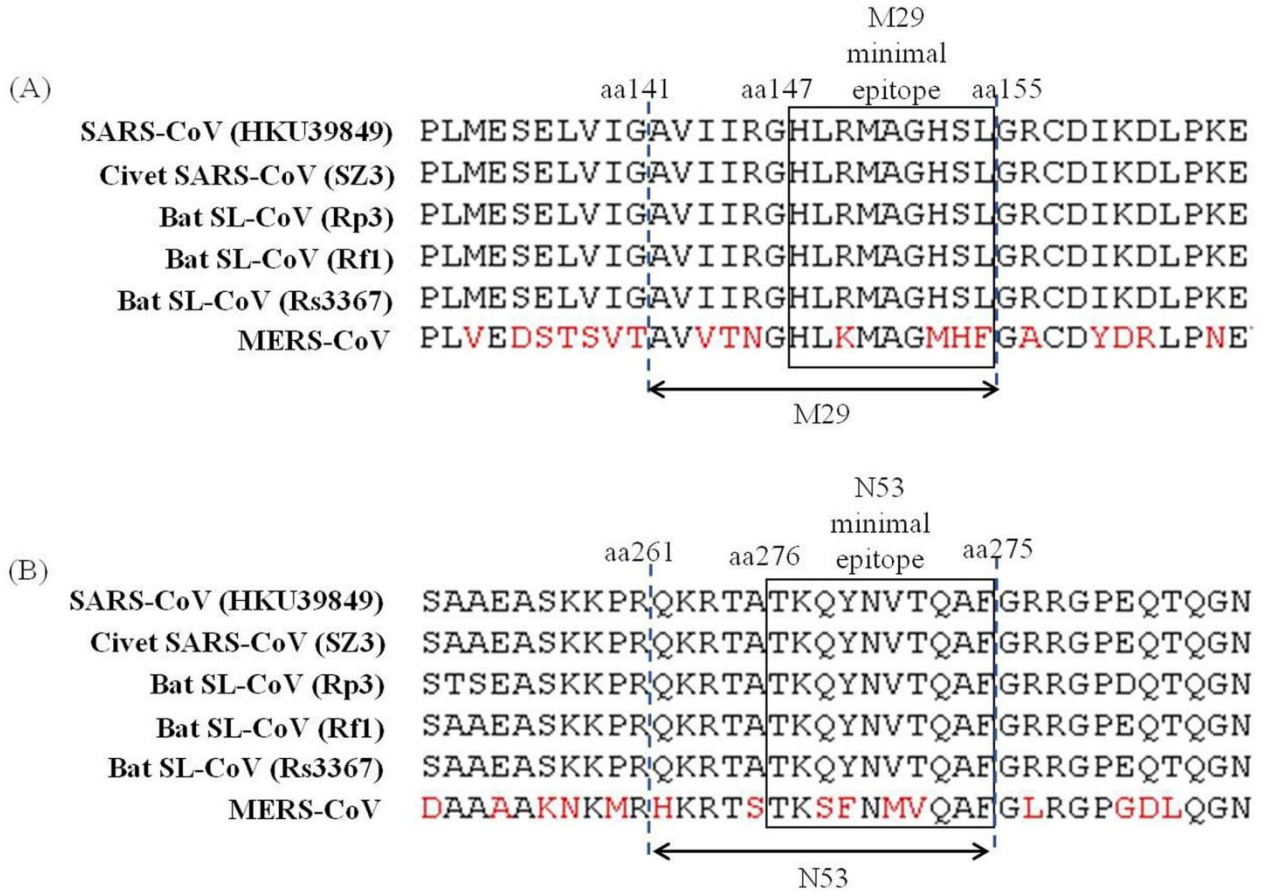

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Supplementary materials

Protein	Matrix (number of numeric pools + number of alphabetic pools)	Number of pools	Total number of peptides in matrix
S	16 + 16	32	249
E / M	7 + 9	16	57 (14 E peptides; 43 M peptides)
N	9 + 9	18	82
3a	7 + 8	15	53
3b / 6	6 + 7	13	40 (29 3b peptides; 11 6 peptides)
7a / 7b	5 + 6	11	30 (23 7a peptides; 7b peptides)
8a / 8b / 9b	6 + 7	13	39 (6 8a peptides; 15 8b peptides; 18 ORF9 peptides)

Supplementary Table 1. Pooling of 550 SARS-CoV peptides spanning the proteome of the structural (S, N, M and E) and accessory (3a, 3b, 6, 7a, 7b, 8a, 8b, 9b) proteins used for ELISpot assay. 15-mer peptides within each protein are arranged in matrices consisting of numeric and alphabetic pools and used for ELISpot screening of memory T cells.

	Clinical presentation	Treatment	Days before fever subsided
SARS subject 1	Patient had hypoxemia and required oxygen supplementation. Patient's chest radiography showed worsening bilateral opacifications. Laboratory abnormalities included elevated levels of aspartate aminotransferase and lactate dehydrogenase and an elevated maximal C-reactive protein level as well as leukopenia and lymphopenia.	Levofloxacin, vancomycin, imipenem, doxycycline, and oseltamivir	17
SARS subject 2	Patient had dry cough and hypoxemia. There were crackles over the patient's lungs. Laboratory abnormalities included an elevated maximal C-reactive protein level, leukopenia and lymphopenia.	Erythromycin and ceftriaxone	17
SARS subject 3	Patient's chest radiography showed a left midzone pulmonary infiltrate and computed tomography of the thorax confirmed the presence of the pulmonary infiltrate in the apical segment of the left lower lobe. Patient's respiratory status remained stable and no supplemental oxygen is required.	Only supportive treatment was given	12

Supplementary Table 2. Clinical information of the SARS-recovered subjects recruited in this study. Some of these information were previously published by Drosten *et al*. (N Engl J Med 2003; 348:1967-1976) and Lim *et al*. (N Engl J Med 2004; 350:1740-1745.).