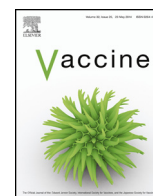




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Memory T cell responses targeting the SARS coronavirus persist up to 11 years post-infection



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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 cellular immunity targeting the viral structural proteins in SARS-recovered individuals is important in the design and development of SARS vaccines, which are currently unavailable.

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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

Abbreviations: SARS, severe acute respiratory syndrome; SARS-CoV, SARS coronavirus.

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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™ (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 IFN γ ELISpot assay

Anti-human IFN γ 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 5×10^4 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 μ g/ml for 5 h in the presence of 10 μ g/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 °C. Cells were washed in 1 \times 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-IFN γ -PE (BD Pharmingen) was carried out at 4 °C, 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 μ g/ml in R10 medium and incubated at 37 °C for 1 h, 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 °C 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 (8–12-mers) spanning the M29 region were tested. For N53 minimal epitope mapping, 6 peptides (8–10-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 IFN γ ELISpot assay using SARS peptide pools arranged in alphabetic and numeric matrices (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 Fig. 1, higher frequencies of IFN γ -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 IFN γ production as identified from ELISpot were further tested by ICS to confirm their abilities to elicit specific T cell IFN γ response and to define the subset of T cells (CD4⁺ or CD8⁺) involved. A total of 4 SARS-specific memory T cell responses were

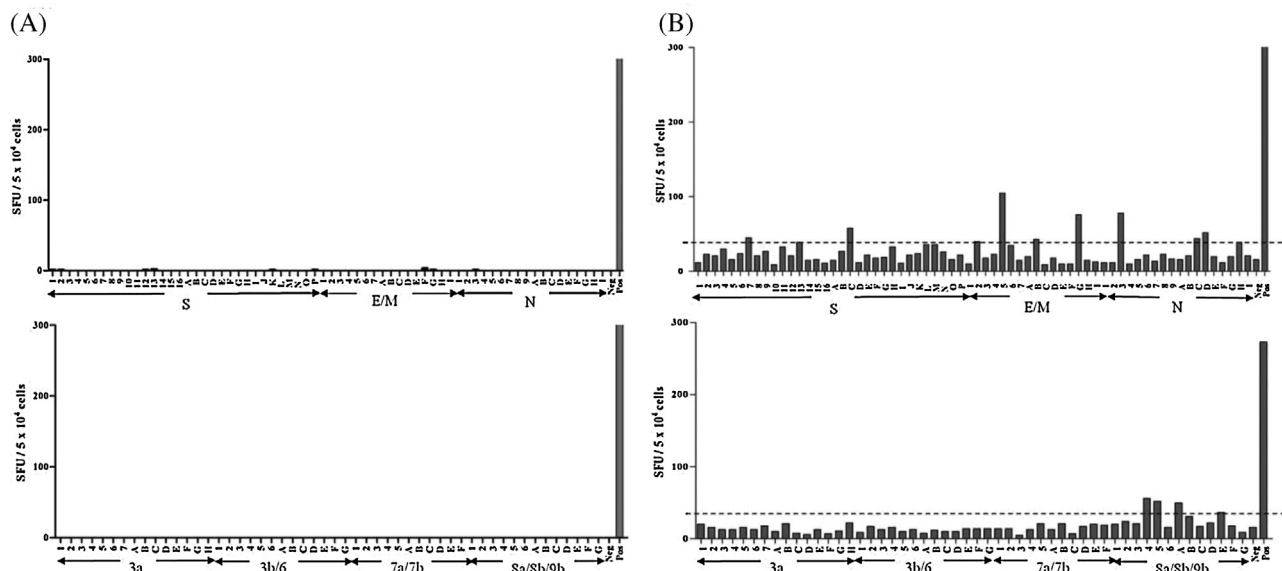


Fig. 1. IFN γ ELISpot results for SARS-specific memory T cell screening. PBMCs from (A) a healthy individual and (B) SARS-recovered individual (SARS subject 1) were expanded *in vitro* using a mixture of SARS-CoV peptides, followed by IFN γ ELISpot assay using SARS peptide matrix pools of the structural (top panels) and accessory proteins (lower panels). Each bar represents the IFN γ -producing response to an individual peptide matrix pool (numeric or alphabetic) in SFU per 5×10^4 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).

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 subjects 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 subjects 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 γ^+ response at 27.6% when stimulated with M29 peptide (Fig. 2, left panels). Additionally, CD107a expression of T cells induced by M29 peptide was determined to be 12.7% (Fig. 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.

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

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

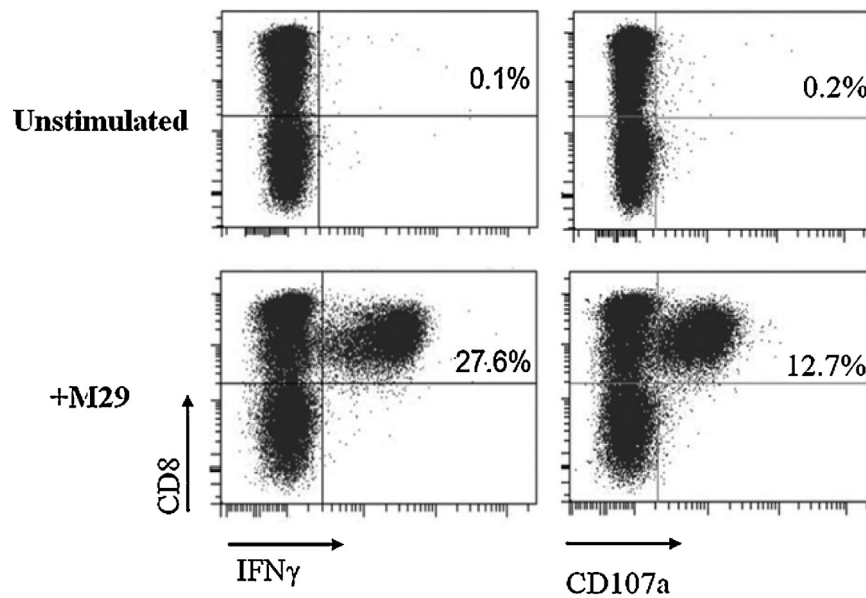


Fig. 2. ICS and flow cytometry analysis of unstimulated and M29-stimulated T cells after restimulation using M29 peptide. The percentages of CD8⁺ IFN γ ⁺ and CD8⁺ CD107a⁺ T cells shown represent the percentage of the T cells in total T cell population (after gating the CD3⁺ cells) present in the short-term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

HLA class I molecules preferentially bind and present peptides of 8–11 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 IFN γ secretion by M29-restimulated T cells. As shown in Table 2, the 9-mer peptide, M29_{147–155}, corresponding to residues 147–155 of M protein, was most efficient in inducing the CD8⁺ T cell response, resulting in the highest percentage of IFN γ -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_{148–155}) or the C-terminus leucine (L) residue (M29_{145–154}) completely abolished IFN γ 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

Table 2

Summary of percentage CD8⁺ IFN γ ⁺ 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 γ ⁺ T cells shown represents the percentage of IFN γ -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 γ ⁺ T cells
M29	15-mer	141–155	AVIIR <i>GHLRM AGHSL</i>	5.3%
M29 _{144–155}	12-mer	144–155	IR <i>GHLRM AGHSL</i>	14.8%
M29 _{143–154}	12-mer	143–154	IIR <i>GHLRM AGHS</i>	0.2%
M29 _{145–155}	11-mer	145–155	R <i>GHLRM AGHSL</i>	17.8%
M29 _{146–155}	10-mer	146–155	<i>GHLRM AGHSL</i>	22.8%
M29 _{145–154}	10-mer	145–154	R <i>GHLRM AGHS</i>	0.2%
M29 _{147–155}	9-mer	147–155	<i>HLRM AGHSL</i>	32.9%
M29 _{148–155}	8-mer	148–155	LRM <i>AGHSL</i>	0.3%
Unstimulated cells				0.2%
PMA/Ionomycin-stimulated cells				11.1%

Table 3
Summary of percentage CD8⁺IFN γ ⁺ 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 γ ⁺ cells shown represent the percentage of IFN γ -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*.

Peptide	Peptide length	Amino acid position	Peptide sequence	Percentage of CD8 ⁺ IFN γ ⁺ T cells
N53	15-mer	261–275	QKRTA <i>TKQYN VTQAF</i>	8.8%
N53 _{266–275}	10-mer	266–275	<i>TKQYN VTQAF</i>	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%

the response (data not shown). It was found that 10-mer peptide, N53_{266–275}, corresponding to residues 266–275 of the N protein, was most efficient in inducing N53T cell response of 12.7% (Table 3). Deletion of N-terminal threonine (T) residue and C-terminal phenylalanine (F) residue from N53_{266–275} led to decrease in percentages of IFN γ -producing CD8⁺ T cells to 10.9% and 8.0%, respectively. This indicates that residues 266–275 are 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 γ ⁺ responses. As shown in Fig. 3 (left panel), when cells were stimulated with M29_{147–155} and N53_{266–275} peptides, CD8⁺IFN γ ⁺ 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

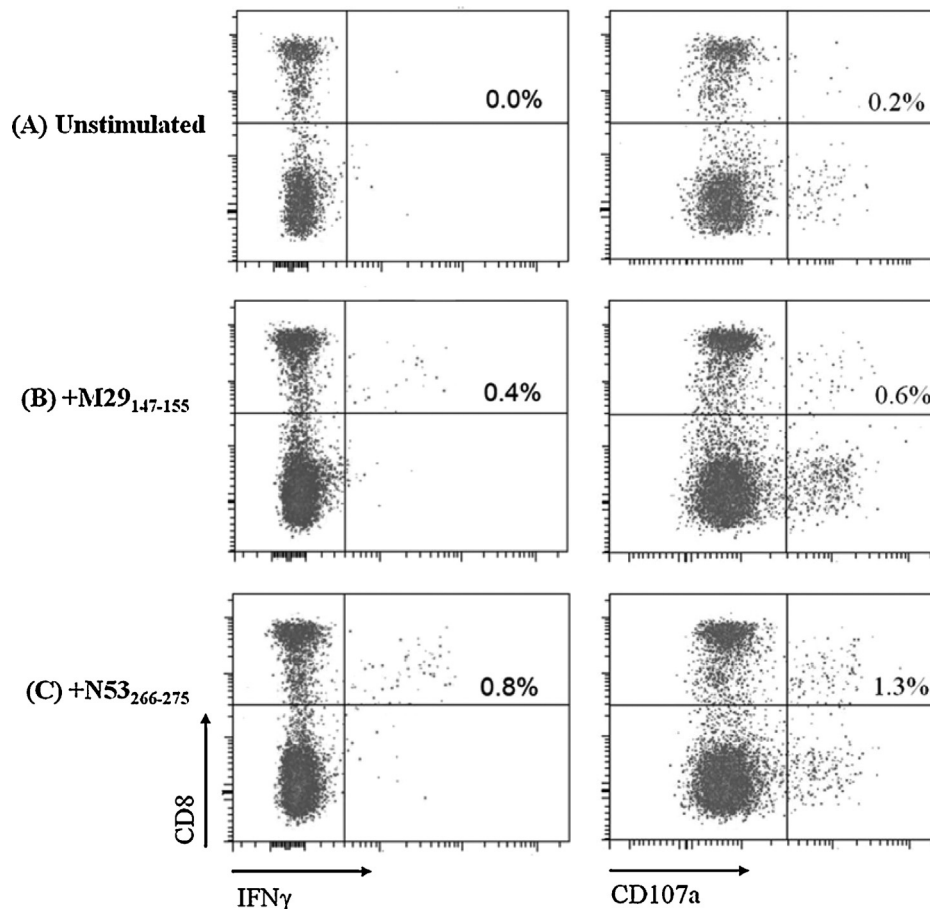


Fig. 3. ICS and flow cytometry analysis of restimulated T cells from SARS subject 1 at 11 years post-infection. Percentages of CD8⁺IFN γ ⁺ responses (left panels) and CD8⁺CD107a⁺ responses (right panels) of (A) unstimulated, (B) M29_{147–155}-stimulated, (C) N53_{266–275}-stimulated T cells are as indicated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

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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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vaccine.2016.02.063>.

References

- [1] Ksiazek TG, Erdman D, Goldsmith CS, Zaki SR, Peret T, Emery S, et al. A novel coronavirus associated with severe acute respiratory syndrome. *N Engl J Med* 2003;348(20 (May)):1953–66.
- [2] Peiris JS, Guan Y, Yuen KY. Severe acute respiratory syndrome. *Nat Med* 2004;10(12 Suppl. (December)):S88–97.
- [3] Li W, Shi Z, Yu M, Ren W, Smith C, Epstein JH, et al. Bats are natural reservoirs of SARS-like coronaviruses. *Science* 2005;310(5748 (October)):676–9.
- [4] Lau SK, Woo PC, Li KS, Huang Y, Tsoi HW, Wong BH, et al. Severe acute respiratory syndrome coronavirus-like virus in Chinese horseshoe bats. *Proc Natl Acad Sci U S A* 2005;102(39 (September)):14040–5.
- [5] Ge XY, Li JL, Yang XL, Chmura AA, Zhu G, Epstein JH, et al. Isolation and characterization of a bat SARS-like coronavirus that uses the ACE2 receptor. *Nature* 2013;503(7477 (November)):535–8.
- [6] Guan Y, Zheng BJ, He YQ, Liu XL, Zhuang ZX, Cheung CL, et al. Isolation and characterization of viruses related to the SARS coronavirus from animals in southern China. *Science* 2003;302(5643 (October)):276–8.
- [7] Rota PA, Oberste MS, Monroe SS, Nix WA, Campagnoli R, Icenogle JP, et al. Characterization of a novel coronavirus associated with severe acute respiratory syndrome. *Science* 2003;300(5624 (May)):1394–9.
- [8] Chen J, Lau YF, Lamirande EW, Paddock CD, Bartlett JH, Zaki SR, et al. Cellular immune responses to severe acute respiratory syndrome coronavirus (SARS-CoV) infection in senescent BALB/c mice: CD4⁺ T cells are important in control of SARS-CoV infection. *J Virol* 2010;84(3 (February)):1289–301.
- [9] Zhao J, Perlman S. T cell responses are required for protection from clinical disease and for virus clearance in severe acute respiratory syndrome coronavirus-infected mice. *J Virol* 2010;84(18 (September)):9318–25.
- [10] Channappanavar R, Fett C, Zhao J, Meyerholz DK, Perlman S. Virus-specific memory CD8T cells provide substantial protection from lethal severe acute respiratory syndrome coronavirus infection. *J Virol* 2014;88(19 (October)):11034–44.
- [11] Li T, Qiu Z, Zhang L, Han Y, He W, Liu Z, et al. Significant changes of peripheral T lymphocyte subsets in patients with severe acute respiratory syndrome. *J Infect Dis* 2004;189(4 (February)):648–51.
- [12] Cameron MJ, Bermejo-Martin JF, Danesh A, Muller MP, Kelvin DJ. Human immunopathogenesis of severe acute respiratory syndrome (SARS). *Virus Res* 2008;133(1 (April)):13–9.
- [13] Oh HL, Chia A, Chang CX, Leong HN, Ling KL, Grotenbreg GM, et al. Engineering T cells specific for a dominant severe acute respiratory syndrome coronavirus CD8T cell epitope. *J Virol* 2011;85(20 (October)):10464–71.
- [14] Case Definitions for Surveillance of Severe Acute Respiratory Syndrome (SARS). <http://www.who.int/csr/sars/casedefinition/en/>. 2003 [Available from: <http://www.who.int/csr/sars/casedefinition/en/>].
- [15] Li T, Xie J, He Y, Fan H, Baril L, Qiu Z, et al. Long-term persistence of robust antibody and cytotoxic T cell responses in recovered patients infected with SARS coronavirus. *PLoS ONE* 2006;1:e24.
- [16] Li CK, Wu H, Yan H, Ma S, Wang L, Zhang M, et al. T cell responses to whole SARS coronavirus in humans. *J Immunol* 2008;181(8 (October)):5490–500.
- [17] Yang LT, Peng H, Zhu ZL, Li G, Huang ZT, Zhao ZX, et al. Long-lived effector/central memory T-cell responses to severe acute respiratory syndrome coronavirus (SARS-CoV) S antigen in recovered SARS patients. *Clin Immunol* 2006;120(2 (August)):171–8.
- [18] Zhou M, Xu D, Li X, Li H, Shan M, Tang J, et al. Screening and identification of severe acute respiratory syndrome-associated coronavirus-specific CTL epitopes. *J Immunol* 2006;177(4 (August)):2138–45.
- [19] Betts MR, Brenchley JM, Price DA, De Rosa SC, Douek DC, Roederer M, et al. Sensitive and viable identification of antigen-specific CD8⁺ T cells by a flow cytometric assay for degranulation. *J Immunol Methods* 2003;281(1–2 (October)):65–78.
- [20] Harding CV, Geuze HJ. Antigen processing and intracellular traffic of antigens and MHC molecules. *Curr Opin Cell Biol* 1993;5(4 (August)):596–605.
- [21] Liu J, Sun Y, Qi J, Chu F, Wu H, Gao F, et al. The membrane protein of severe acute respiratory syndrome coronavirus acts as a dominant immunogen revealed by a clustering region of novel functionally and structurally defined cytotoxic T-lymphocyte epitopes. *J Infect Dis* 2010;202(8 (October)):1171–80.
- [22] Fan YY, Huang ZT, Li L, Wu MH, Yu T, Koup RA, et al. Characterization of SARS-CoV-specific memory T cells from recovered individuals 4 years after infection. *Arch Virol* 2009;154(7):1093–9.
- [23] Tsao YP, Lin JY, Jan JT, Leng CH, Chu CC, Yang YC, et al. HLA-A*0201 T-cell epitopes in severe acute respiratory syndrome (SARS) coronavirus nucleocapsid and spike proteins. *Biochem Biophys Res Commun* 2006;344(1 (May)):63–71.
- [24] Kim TW, Lee JH, Hung CF, Peng S, Roden R, Wang MC, et al. Generation and characterization of DNA vaccines targeting the nucleocapsid protein of severe acute respiratory syndrome coronavirus. *J Virol* 2004;78(9 (May)):4638–45.
- [25] Rivino L, Tan AT, Chia A, Kumaran EA, Grotenbreg GM, MacAry PA, et al. Defining CD8⁺ T cell determinants during human viral infection in populations of Asian ethnicity. *J Immunol* 2013;191(8 (October)):4010–9.
- [26] Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus AD, Fouchier RA. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. *N Engl J Med* 2012;367(19 (November)):1814–20.
- [27] de Groot RJ, Baker SC, Baric RS, Brown CS, Drosten C, Enjuanes L, et al. Middle East respiratory syndrome coronavirus (MERS-CoV): announcement of the Coronavirus Study Group. *J Virol* 2013;87(14 (July)):7790–2.
- [28] Chan JF, Lau SK, To KK, Cheng VC, Woo PC, Yuen KY. Middle East respiratory syndrome coronavirus: another zoonotic betacoronavirus causing SARS-like disease. *Clin Microbiol Rev* 2015;28(2 (April)):465–522.
- [29] Cheng VC, Lau SK, Woo PC, Yuen KY. Severe acute respiratory syndrome coronavirus as an agent of emerging and reemerging infection. *Clin Microbiol Rev* 2007;20(4 (October)):660–94.
- [30] van Boheemen S, de Graaf M, Lauber C, Bestebroer TM, Raj VS, Zaki AM, et al. Genomic characterization of a newly discovered coronavirus associated with acute respiratory distress syndrome in humans. *MBio* 2012;3(6).
- [31] Martin JE, Louder MK, Holman LA, Gordon IJ, Enama ME, Larkin BD, et al. A SARS DNA vaccine induces neutralizing antibody and cellular immune responses in healthy adults in a Phase I clinical trial. *Vaccine* 2008;26(50 (November)):6338–43.