Human CD4$^+$ memory T-lymphocyte responses to SARS coronavirus infection

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Abstract

Little is known about CD4$^+$ T-cell immunity to the severe acute respiratory syndrome (SARS) coronavirus. In two SARS patients, we examined the memory CD4$^+$ T-cell responses to peptides derived from SARS coronavirus structural proteins. We generated CD4$^+$ T-cell lines to 3 peptides from the spike (S) protein and defined their HLA restriction. In one patient, the predominant memory CD4$^+$ T-cell response was directed against an epitope outside the S protein receptor-binding domain. In both patients, the frequency of CD4$^+$ memory T-cells to virus structural proteins and anti-SARS coronavirus IgG levels were low by 12 months after infection. This report expands our understanding of the specificity and duration of anti-SARS coronavirus CD4$^+$ T-cell immune responses.
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The severe acute respiratory syndrome (SARS) is caused by the SARS coronavirus (SARS CoV), and emerged from a zoonotic origin in late 2002 in southern China (Drosten et al., 2003). Human-to-human transmission spread SARS to 29 countries. The epidemic ended in July 2003 and produced 8098 cases with an overall 9.6% case-fatality rate (2006). Re-emergence of SARS CoV transmission in humans is not likely but remains possible. The potential for other zoonotic coronaviruses to jump to humans and cause serious illness also exists. Ideally, vaccine candidates for SARS or SARS-like coronaviruses should induce robust humoral and cell-mediated immune responses. Neutralizing antibodies to SARS CoV are directed against epitopes in the virus spike (S) structural protein, and these antibodies elicit protective immunity in animal models of SARS (Greenough et al., 2005; Roberts et al., 2006; Subbarao et al., 2004). Less is known about T-cell immunity to SARS CoV infection, particularly CD4$^+$ T-helper immunity that could promote protective antibody development.

We undertook a study to characterize T-cell responses directed against the structural proteins of SARS CoV from two healthcare workers who recovered from SARS. Our approach was to first identify peptides that stimulated interferon (IFN)-γ responses from memory T-cells in as unbiased a fashion as possible. We generated and characterized CD4$^+$ T-cell lines to 3 peptides, and measured the circulating frequencies of peptide-specific memory T-cells 6–30 months after infection.

Results and discussion

Peptide pool screening of peripheral blood mononuclear cells by IFN-γ ELISPOT

Peripheral blood mononuclear cells (PBMCs) collected 6 months after SARS CoV infection were stimulated in IFN-γ ELISPOT assays with overlapping peptide pools spanning the SARS CoV structural proteins (envelope (E), matrix (M), nucleocapsid (N), and S proteins) (11–12 peptides/pool, peptide concentration=4 μg/ml). In the PBMC from one recovered SARS patient (SARS patient #1 – HLA-DR03$^+$08$^+$), there were 17 peptide pools that produced ≥25 IFN-γ spot-forming cells

(SFC)/10^6 PBMC (range 25–70 IFN-γ SFC /10^6 PBMC). In PBMC from the other recovered SARS patient (SARS patient #2 – HLA-DR15$^+$), there were no peptide pools that produced ≥ 25 IFN-γ SFC/10^6 PBMC even though the response to phytohemagglutinin was robust. We further stimulated and expanded SARS patient #2 PBMC *in vitro* with a pool of eight peptides that contained predicted high affinity HLA-DRB1*1501 epitopes (S$_{1–18}$, S$_{307–322}$, S$_{313–329}$, M$_{14–31}$, M$_{22–38}$, M$_{90–106}$, E$_{9–26}$, E$_{17–34}$) (Singh and Raghava, 2001; Rammensee et al., 1999). Memory CD4$^+$ T-cells directed against this peptide pool were detected by IFN-γ intracellular cytokine staining (ICS), but only after two rounds of *in vitro* re-stimulation and expansion (individual peptide concentration=10 μg/ml) (% IFN-γ-producing CD3$^+$CD4$^+$ T-cells: SARS patient #2 – 2.6%, HLA-DR15$^+$ SARS seronegative control – 0.06%).

Since SARS patient #1 had measurable memory CD4$^+$ T-cell responses *ex vivo*, we stimulated patient #1 PBMC with 40 different individual peptides taken from the 17 positive pools in an IFN-γ ELISPOT assay (peptide concentrations=10 μg/ml). We selected the five peptides with the highest IFN-γ SFC frequencies (range 14–21/10^6 PBMC) for further study. These five peptides were all from the viral S protein.

Generation of T-cell lines

The five S protein-derived peptides were pooled and used for bulk culture stimulation of convalescent PBMC from SARS patient #1. 6 T-cell lines directed against 3 peptides (S$_{729–745}$, S$_{358–374}$, S$_{427–444}$) were successfully established (Table 1). Four CD4$^+$ T-cell lines recognized peptide S$_{729–745}$ (TECANLLL-QYGSFCTQL). 3 of these 4 lines were clearly HLA-DR-restricted, and CTL activity against allogeneic target cells suggested HLA-DR08 restriction. The optimal epitope for HLA-DR08 restricted CD4$^+$ T-cell activation was essentially the entire 17mer peptide. By IFN-γ ICS (10 μg/ml pep-

tide×6 h), only peptide S$_{729–745}$, and not the flanking overlapping peptides S$_{723–738}$ or S$_{736–753}$, stimulated IFN-γ production from the four CD4$^+$ T-cell lines noted above (Fig. 1). The highest HLA-DR08 gene frequencies have been reported in Latino American and Asian American racial groups, and HLA-DR08 alleles have been reported at moderate frequencies in Filipino populations (Mack et al., 2000). Peptide S$_{358–374}$ (STFFSTFKCYGVSATKL) also appeared to be HLA-DR08 restricted. The HLA restriction of peptide S$_{427–444}$ (NIDATSTGNYNYKYRYLR) was unable to be determined (Table 1). Neutralizing antibodies to the SARS CoV recognize epitopes in the receptor-binding domain (RBD) of the S protein – between amino acids 318 and 510 (He et al., 2005; Hwang et al., 2006; Li et al., 2005). Two of the three S protein CD4$^+$ T-cell epitopes we identified are in the RBD (S$_{358–374}$ and S$_{427–444}$). However, the predominant CD4$^+$ T-cell response in recovered SARS patient #1 was directed against an epitope outside the RBD (S$_{729–745}$).

Frequencies of SARS CoV peptide-specific memory CD4$^+$ T-cells

The frequency of IFN-γ^+ SFC to peptide S$_{729–745}$ was greatest 6 months after SARS CoV infection in patient #1, and decreased to near the limit of detection by IFN-γ ELISPOT 12 months onward ($\leq 5/10^6$ PBMC) (Fig. 2a). S$_{729–745}$-specific CTL activity could still be detected after *in vitro* re-stimulation of SARS patient #1 PBMC collected at 12 months (7.6% S$_{729–745}$-specific lysis), but not at 24 and 30 months. The waning of S$_{729–745}$-specific memory T-cells paralleled the waning of anti-SARS CoV IgG levels in SARS patient #1. Anti-SARS CoV IgG levels were already 4-fold lower in patient #2 than patient #1 at 6 months (Fig. 2b). Anti-SARS CoV IgG and memory CD4$^+$ T-cell responses were weaker or waned more rapidly in patient #2 compared to patient #1.

Table 1
Characteristics of T-cell lines generated from convalescent PBMC of SARS patient #1

Cell line	Peptide specificity	% CD4^{+} [a]	CD3$^+$CD4$^+$ IFN-γ^+ cells [b]	HLA restriction [c]	CTL activity to peptide-loaded target cells (% specific lysis) [d]		
					Autologous HLA-DR03$^+$08$^+$ B-LCL	Allogeneic HLA-DR03$^+$ B-LCL	Allogeneic HLA-DR08$^+$ B-LCL
C4	S$_{729–745}$ (TECANLLLQYGSFCTQL)	98%	Yes	HLA-DR	57%	3%	52%
F2	S$_{729–745}$	96%	n.d.	HLA-DR	66%	−1%	32%
G7	S$_{729–745}$	98%	Yes	HLA-DR	54%	3%	14%
F7	S$_{729–745}$	97%	Yes	Not blocked by mAbs to HLA-DR, -DP, -DQ, or -ABC	72%	−2%	29%
F5	S$_{358–374}$ (STFFSTFKCYGVSATKL)	98%	Yes	HLA-DR	31%	0%	14%
B4	S$_{427–444}$ (NIDATSTGNYNYKYRYLR)	85%	n.d.	n.d.	35%	0%	2%

[a] Cell lines were surface-stained with APC-Cy7.7-conjugated anti-CD3, Alexa405-conjugated anti-CD4, and APC-Cy5.5-conjugated anti-CD8 monoclonal antibodies (mAbs) and analyzed on a FACSAria™ flow cytometer (BD Biosciences, San Jose, CA); values are % of CD3$^+$ lymphocytes that were also CD4$^+$.

[b] Cell lines were stimulated with the specified peptide in an IFN-γ ICS assay; results are whether the IFN-γ^+ cells in the cell line were CD3$^+$CD4$^+$; n.d.=not done.

[c] HLA restriction was determined by inhibition of cytotoxic activity in standard ^{51}Cr release CTL assays in the presence of blocking mAbs to the HLA haplotype compared to isotype control mAbs; n.d.=not done.

[d] Standard ^{51}Cr release cytotoxic T lymphocyte (CTL) assays were performed using autologous and allogeneic B-lymphoblastoid cell lines (B-LCLs) as target cells; values are [% lysis of peptide-loaded B-LCLs−% lysis of B-LCLs alone] (mean of triplicates); results using allogeneic B-LCL targets are representative of at least 2 separate experiments using 2 different allogeneic donor cell lines.
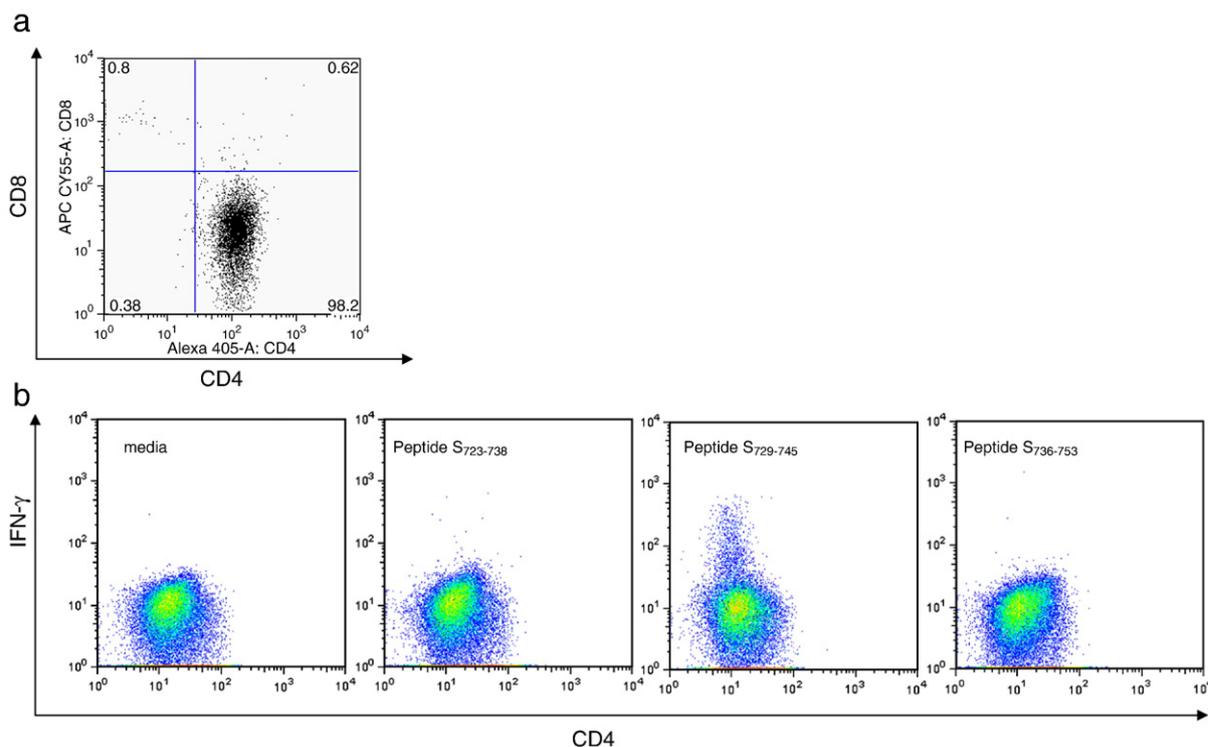
Fig. 1. Peptide $S_{729-745}$ from the SARS CoV spike (S) protein contains a CD4$^+$ T-cell epitope. (a) The G7 cell line (Table 1) was surface-stained with APC-Cy7-conjugated anti-CD3, Alexa405-conjugated anti-CD4, and APC-Cy5.5-conjugated anti-CD8 mAbs and gated on CD3$^+$ lymphocytes. (b) The G7 cell line was stimulated with peptide $S_{723-738}$ (YICGDSTECANLLLQY), peptide $S_{729-745}$ (TECANLLLQYGSFCTQL), or peptide $S_{736-753}$ (LQYGSFCTQLNRALSGIA) (10 μg/ml×6 h) in an IFN-γ ICS assay. Cells were gated on CD3$^+$CD14/19/56$^-$CD8$^-$ lymphocytes. Results are representative of the findings in the other $S_{729-745}$-specific T-cell lines.

The $S_{729-745}$-specific memory CD4$^+$ T-cell frequency in SARS patient #1 measured by IFN-γ ELISPOT and ICS was 0.003–0.004%. It was notably higher than in SARS patient #2 (HLA-DR15$^+$) and 7 seronegative close contacts including 2 that were HLA-DR08$^+$ (Fig. 2c). An epitope-specific CD4$^+$ T-cell frequency of 0.003–0.004% 6 months after infection is approximately 10-fold lower than what has been reported 1 month after natural influenza virus infection in adults (Linnemann et al., 2000). While influenza and SARS are both respiratory virus infections, the CD4$^+$ T-cell response to influenza in adults is a secondary one, compared to a primary CD4$^+$ T-cell response for SARS.

Even though we generated CD4$^+$ T-cell lines to peptides $S_{358-374}$ and $S_{427-444}$ from patient #1 PBMC, the direct ex vivo circulating frequencies of memory T-cells to these peptides were low and near the limit of detection by IFN-γ ELISPOT 6–30 months after infection ($\leq 8/10^6$ PBMC). The low $S_{358-374}$ and $S_{427-444}$-specific memory T-cell frequencies in SARS patient #1 were not different than in SARS patient #2 or 7 seronegative close contacts (Fig. 2c). CD4$^+$ T-cell responses to peptides $S_{358-374}$ and $S_{427-444}$ were weaker or decreased more rapidly than the CD4$^+$ T-cell response to $S_{729-745}$ in SARS patient #1. CD4$^+$ T-cell responses to any SARS CoV structural protein epitopes were weaker or decreased more rapidly in SARS patient #2 compared to patient #1. However, our results cannot determine what levels of peptide-specific CD4$^+$ memory T-cells or anti-SARS CoV IgG are associated with effective SARS CoV immunity.

One other group has measured SARS CoV-specific CD4$^+$ memory T-cell responses in recovered SARS individuals (Peng et al., 2006a,b; Yang et al., 2006). Individual CD4$^+$ T-cell epitopes were identified within the N protein (Peng et al., 2006b). We believe our study is the first to identify individual human CD4$^+$ T-cell epitopes within the SARS CoV S protein and characterize their HLA restriction (HLA-DR08). We have also identified potential HLA-DR15-restricted peptides from the SARS CoV S, M, and E proteins. Some have suggested that the SARS CoV S protein RBD by itself could be an effective subunit vaccine (He et al., 2005). Our findings suggest that dominant SARS CoV anti-S CD4$^+$ T-helper responses could be directed to epitopes outside the RBD. Our data also illustrate that in some individuals humoral and CD4$^+$ T-cell immunity to SARS CoV may wane rapidly. What levels of humoral or CD4$^+$ T-cell immunity are associated with clinical protection remain unknown. An improved understanding of SARS CoV-specific memory immune responses will help guide future vaccination strategies in the event of SARS or SARS-like coronavirus re-emergence in humans.

Materials and methods

Subjects and source of cells

A limited outbreak of SARS occurred in the Philippines in April 2003 (2003). We collected serial convalescent blood
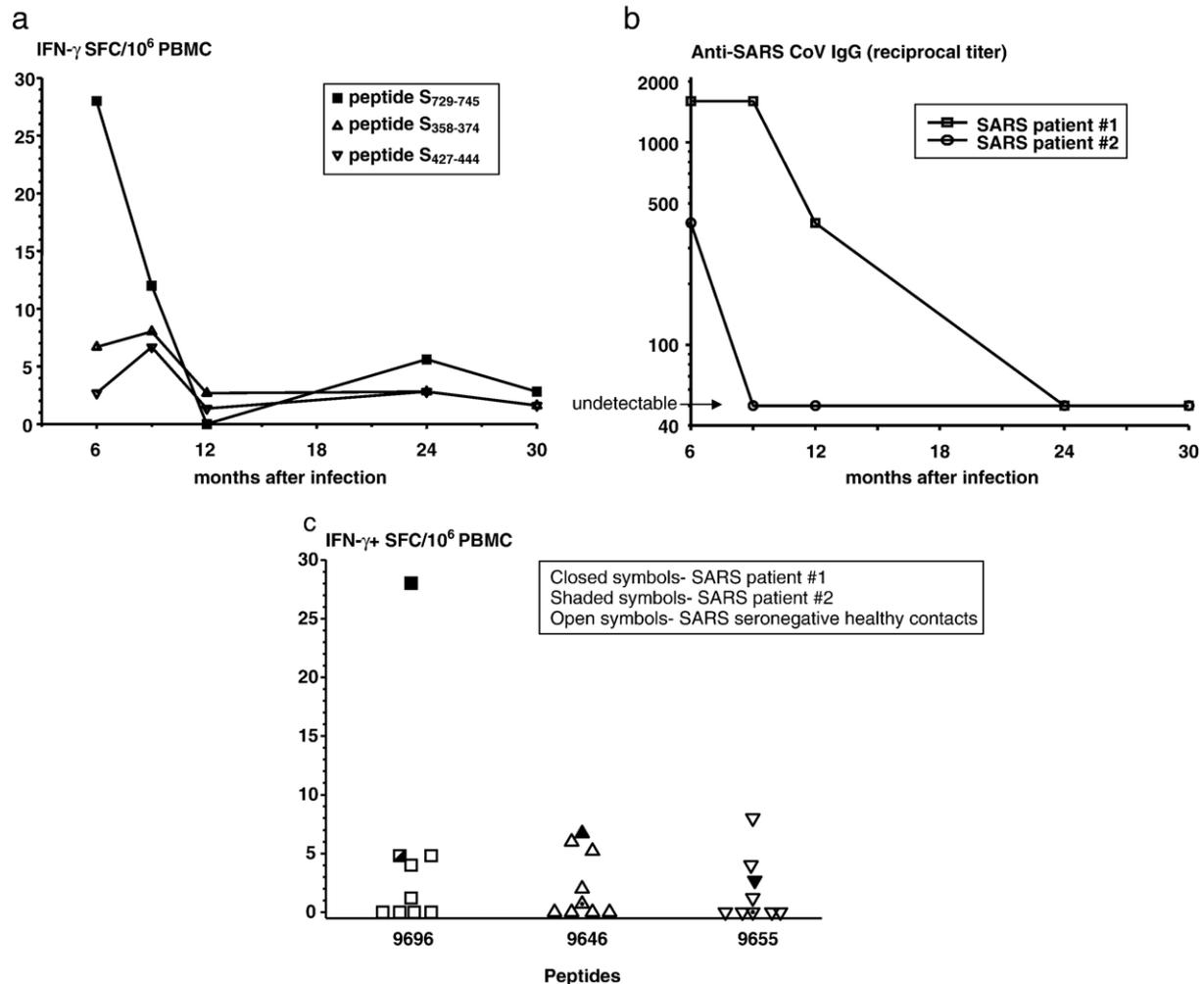
Fig. 2. Peptide-specific T-cell frequencies and anti-SARS CoV IgG titers in recovered SARS patients. (a) Serial convalescent PBMC collected from SARS patient #1 were stimulated with peptides $S_{729–745}$, $S_{358–374}$, and $S_{427–444}$ in an IFN-γ ELISPOT assay (peptide concentration = 10 μg/ml). Results are expressed as number of peptide-specific IFN-γ spot-forming cells (SFC)/10^6 PBMC (media background subtracted, mean of triplicate values). (b) Anti-SARS CoV IgG titers were measured by ELISA to inactivated virus-infected cell lysates in serial plasma samples collected from SARS patients #1 and #2. Fourfold serial dilutions of each plasma sample were assayed in duplicate. Reciprocal titer < 100 was undetectable (coded as reciprocal titer = 50). (c) PBMC collected 6 months after the SARS outbreak from the two SARS patients and 7 SARS seronegative healthy contacts were stimulated with peptides $S_{729–745}$, $S_{358–374}$, and $S_{427–444}$ in an IFN-γ ELISPOT assay (peptide concentration = 10 μg/ml). Two healthy contacts were HLA-DR08+. Results are expressed as number of peptide-specific IFN-γ SFC/10^6 PBMC (media background subtracted, mean of triplicate values).

samples from two healthcare workers who contracted SARS in the outbreak and recovered, and from 16 healthy contacts. Peripheral blood mononuclear cells (PBMC) and plasma were aliquoted and cryopreserved. Red blood cell (RBC) pellets containing granulocytes were used for HLA typing (Biotest ABDR SSP kit, Biotest Diagnostics Corp., Denville, NJ). All participants provided written informed consent, and the protocol was approved by the Institutional Review Boards of the University of Massachusetts Medical School and the Research Institute for Tropical Medicine.

Reagents

A total of 266 peptides spanning the SARS CoV envelope, matrix, nucleocapsid, and spike proteins were provided by NIH/NIAID *BEI*Resources Repository (Cat# 9900–9903). The peptides were 15–17 amino acids in length, overlapping by 10 amino acids.

Assays

IFN-γ ELISPOT assays were performed as previously described, without addition of co-stimulatory antibodies or interleukin-2 (Jameson et al., 1998). T-cell lines were established by bulk culture peptide stimulation, limiting dilution, and standard ^{51}Cr release CTL assays (Jameson et al., 1998). IFN-γ ICS assays were performed after 6-h stimulation with peptide in the presence of a Golgi inhibitor (brefeldin A), as previously described (Mangada et al., 2004). Anti-SARS CoV IgG titers were measured by ELISA to inactivated virus-infected cell lysates in serial plasma samples, as previously described (Ksiazek et al., 2003). Fourfold serial dilutions of each plasma

sample were assayed in duplicate. A reciprocal titer <100 was undetectable (coded as reciprocal titer=50). All 16 healthy contacts had undetectable anti-SARS CoV IgG levels (reciprocal titer <100).

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