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Response of Memory CD8$^+$ T Cells to Severe Acute Respiratory Syndrome (SARS) Coronavirus in Recovered SARS Patients and Healthy Individuals[1]

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To date, the pathogenesis of severe acute respiratory syndrome (SARS) in humans is still not well understood. SARS coronavirus (SARS-CoV)-specific CTL responses, in particular their magnitude and duration of postinfection immunity, have not been extensively studied. In this study, we found that heat-inactivated SARS-CoV elicited recall CTL responses to newly identified spike protein-derived epitopes (SSp-1, S978, and S1202) in peripheral blood of all HLA-A*0201$^+$ recovered SARS patients over 1 year postinfection. Intriguingly, heat-inactivated SARS-CoV elicited recall-like CTL responses to SSp-1 but not to S978, S1202, or dominant epitopes from several other human viruses in 5 of 36 (13.8%) HLA-A*0201$^+$ healthy donors without any contact history with SARS-CoV. SSp-1-specific CTLs expanded from memory T cells of both recovered SARS patients, and the five exceptional healthy donors shared a differentiated effector CTL phenotype, CD45RA$^+$CCR7$^-$CD62L$^-$, and expressed CCR5 and CD44. However, compared with the high avidity of SSp-1-specific CTLs derived from memory T cells of recovered SARS patients, SSp-1-specific CTLs from the five exceptional healthy donors were of low avidity, as determined by their rapid tetramer dissociation kinetics and reduced cytotoxic reactivity, IFN-γ secretion, and intracellular production of IFN-γ, TNF-α, perforin, and granzyme A. These results indicate that SARS-CoV infection induces strong and long-lasting CTL-mediated immunity in surviving SARS patients, and that cross-reactive memory T cells to SARS-CoV may exist in the T cell repertoire of a small subset of healthy individuals and can be reactivated by SARS-CoV infection. ***The Journal of Immunology,*** **2005, 175: 591–598.**

The first recorded outbreak of severe acute respiratory syndrome (SARS)[3] in late February 2003 has led to thousands of infected patients and hundreds of deaths. The etiological agent of the syndrome, a novel coronavirus termed SARS coronavirus (SARS-CoV), has since been identified and isolated (1–4), and its genome has been sequenced (5–7).

To date, the pathogenesis of SARS in humans is still not well understood. In particular, the role of cell-mediated immunity on SARS-CoV infection is still not clear. SARS-associated lymphopenia has also been well documented (8–10), but the underlying mechanism remains unknown. Some previous studies demonstrated the absolute counts of lymphocytes and lymphocyte subsets to be a useful indicator in the development of effective methods of diagnosis and treatment of SARS (11, 12), and three HLA-A*0201-restricted CD8$^+$ T cell epitopes, SSp-1, S978, and S1202, were recently identified from SARS-CoV spike protein (13–15).

Although memory T cell responses to epitopes S1202 and S978 were detected in an IFN-γ ELISPOT assay in PBMCs from HLA-A*0201$^+$ recovered SARS patients up to 3 mo postinfection (14, 15), how long this immunity will last requires further monitoring. Surprisingly, in the generation of effector CTLs from healthy donors without any contact history with SARS-CoV following in vitro repeated challenge of PBMCs with heat-inactivated SARS-CoV-pulsed dendritic cells in culture medium supplemented with IL-7, IL-10, and IL-2, inactivated SARS-CoV provoked recall-like T cell responses in certain healthy donors, which hints that there might exist memory T cell populations cross-reactive to SARS-CoV. It is attractive to us to elucidate these questions. The recently identified epitopes, SSp-1, S978, and S1202, and HLA-A*0201/SSp-1 tetramers (13) help quantitatively and qualitatively investigate characteristics of specific memory T cell responses to heat-inactivated SARS-CoV in recovered SARS patients over 1 year postinfection and in healthy individuals.

Materials and Methods

Donors

SARS-CoV-seropositive blood samples were taken from recovered patients (12–14 mo after recovery) with their informed consent and the approval of the relevant local hospital ethical committees in Guangzhou, China. Healthy donor blood samples were obtained from the Shanghai Blood Center. All of the donors were from 18 to 61 years of age. The 13 recovered SARS patients comprising 8 females and 5 males selected for

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[3] Abbreviations used in this paper: SARS, severe acute respiratory syndrome; SARS-CoV, SARS coronavirus; SSp-1, SARS-CoV spike protein-derived CD8$^+$ T cell epitope 1; SFC, spot-forming cell.

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this study were typed as HLA-A*0201 subtype positive by PCR-based DNA typing (16). Twelve HLA-A*0201$^-$ recovered SARS patients comprising 5 females and 7 males, and 36 HLA-A*0201$^+$ healthy donors comprising 21 females and 15 males without any contact history acted as the controls. Abs against SARS-CoV were detected by SARS-specific IgG capture ELISA and end-point dilution Ab titers, ranging from 1:8 to 1:64, and were observed in all recovered SARS patients. No Ab to SARS-CoV was detected in any healthy donors.

Peptides

HLA-A*0201-restricted SARS-CoV spike protein-derived CD8$^+$ T cell epitopes, SSp-1 (RLNEVAKNL, residues 1167–1175), S978 (LITGRLQSL, residues 978–986), S1202 (FIAGLIAIV, residues 1202–1210), epitopes from HCoV-229E spike protein (H77, LLLNCLWSV, residues 77–85; H881, LITGRLAAL, residues 881–889), and dominant epitopes from human influenza A virus matrix protein (GILGFVFTL, residues 58–66), human CMV pp65 (NLVPMVATV, residues 495–503), EBV LMP2 (CLGGLLTMV, residues 426–434), hepatitis B virus core Ag (FLPSD FFPSV, residues 18–27), carcinoembryonic Ag (CAP-1, YLSGANLNL, residues 571–579), were synthesized at GL Biochem and purified to >98% by reverse-phase HPLC, as confirmed by mass spectrometry. Lyophilized peptides were dissolved in DMSO and stored in aliquots at −80°C until used.

Cell lines and spike protein-recombinant adenovirus transfection

Human TAP-deficient T2, embryonic kidney 293, colorectal carcinoma SW480 (HLA-A2.1$^+$), and HT29 (HLA-A2.1$^-$) cell lines were obtained from the American Type Culture Collection. Spike protein-transfected target cells were prepared by us as previously described (13). Briefly, spike protein cDNA was amplified by RT-PCR using forward (5′-GCCTC GAGACCATGTTTATTTTCTTATTATTTCTTACTCTCACTAGTGGTAG TGACCT-3′) and reverse (5′-TTTATGTGTAATGTAATTTGACACC CTT-3′) primers from RNA extracted from the SARS-CoV BJ01 strain (7). Recombinant adenoviruses containing spike protein cDNA were constructed and propagated in 293 cells, with virus titers determined by plaque assay on 293 cells. For infection, SW480 and HT29 cells were incubated with recombinant adenovirus at a multiplicity of infection of 50:1 for 24 h. Spike protein expression was confirmed by RT-PCR and Western blot using spike protein-specific Ab (IMGENEX). SW480 and HT29 cells transfected with recombinant adenovirus containing β-galactosidase cDNA were used as controls.

Virus and virus inactivation

The SARS-CoV BJ01 strain (7) was propagated in Vero cells cultured in IMDM (HyClone). The viral suspension was centrifuged at 300 × *g* for 10 min, and the supernatant was aliquoted and stored at −80°C. The virus stock titer was 1.6×10^7 median tissue culture-infective doses ($TCID_{50}$) per milliliter. To inactivate, virus stock was heated at 60°C for 60 min, and viral particles were purified by chromatographic separation. All cell cultures were performed in biosafety level 3 conditions.

CTL expansion and cloning from memory CD8$^+$ T cells

PBMCs were isolated from whole blood of recovered SARS patients or healthy donors by Ficoll-Hypaque (Sigma-Aldrich) density gradient centrifugation. A total of 2×10^6/ml PBMCs was challenged with heat-inactivated SARS-CoV (1.6×10^6 $TCID_{50}$ per milliliter) in RPMI 1640 medium containing 10% heat-inactivated FCS and 20 IU/ml rIL-2 (Sigma-Aldrich). Cultured cells were collected periodically for flow cytometry analysis. For functional assessment, tetramer-positive CD8$^+$ lymphocytes were positively selected by tetramer magnetic cell sorting from cell culture (17, 18). Thirty million cultured PBMCs were stained with 1 μg of PE-conjugated HLA-A*0201/SSp-1 tetramer for 30 min at room temperature. Cells were washed twice and then incubated with 2 μg of anti-PE MicroBeads (Miltenyi Biotec) for 20 min at 4°C. The cells were then washed once with PBS, applied to MS separator columns (Miltenyi Biotec), and positive cells were separated according to the manufacturer's instructions. Isolated tetramer-positive CTLs were cloned by limiting dilution and maintained in RPMI 1640 medium supplemented with 10% human AB serum and 50 IU/ml rIL-2 in the presence of irradiated (80 Gy) allogeneic PBMCs as feeder cells (19).

Tetramer staining and flow cytometry immunofluorescence analysis

HLA-A*0201/SSp-1 tetramers were constructed as previously described by us (13). Cells were first incubated with PE-conjugated tetramers for 1 h at 37°C, and then FITC-conjugated anti-CD8, -CD45RA, -CCR7, -CD62L, -CCR5, or -CD44 mAbs (BD Pharmingen; Biolegend) were added, and cells were incubated for an additional 30 min at 4°C. For intracellular cytokine staining, short-term virus-challenged PBMCs from patients and healthy donors were restimulated for 4 h in the presence or absence of 20 μM SSp-1. Brefeldin A (Sigma-Aldrich), at a final concentration of 20 μg/ml, was added to enable intracellular proteins to accumulate. Anti-IFN-γ, -TNF-α, -perforin, or -granzyme A mAbs (BD Pharmingen; Biolegend) were added after cells were fixed and permeabilized using saponine (Sigma-Aldrich). After washing with PBS, stained cells were fixed with 0.5% paraformaldehyde and analyzed by flow cytometry (FACScan or FACSVantage SE; BD Biosciences). Data analysis was performed using CellQuest software.

Cytotoxicity assay

CTLs were tested for cytotoxicity in a standard 4-h ^{51}Cr release assay as previously described by us (13). Each assay was performed in triplicate. Percent specific lysis was determined according to the following formula: percent specific lysis = [(mean experimental cpm − mean spontaneous cpm)/(mean maximum cpm − mean spontaneous cpm)] × 100%. Spontaneous and maximum releases were determined by incubating the labeled targets with medium alone or 2% Triton X-100, respectively. Spontaneous release was always <15% of maximum release. The SD of triplicate wells was <10%.

ELISPOT assay

ELISPOT assays were performed using a commercially available kit (R&D Systems) according to the manufacturer's instructions. Resulting spots were counted using a stereomicroscope (Carl Zeiss) under ×40 magnification. Only brown colored spots with fuzzy borders were scored as spot-forming cells (SFC). The data in the figures refer to the means of triplicate assays. SD was generally within 10% of the mean.

Statistical analysis

Student's *t* test analysis was used to determine the significance of the results. Values of $p \leq 0.05$ indicated statistical significance.

Results

Memory CTL responses in recovered SARS patients

Our investigation into cell-mediated immunity in recovered SARS patients first focused on identifying IFN-γ-secreting T cells and HLA-A*0201/SSp-1 tetramer-positive CD8$^+$ T cells in peripheral blood. Although memory T cell responses to epitopes S1202 and S978 were detected in an IFN-γ ELISPOT assay in PBMCs from HLA-A*0201$^+$ recovered SARS patients up to 3 mo postinfection (14, 15), we found no evidence of IFN-γ-secreting T cells to SSp-1, S978, or S1202, or HLA-A*0201/SSp-1 tetramer-positive CD8$^+$ T cells among freshly isolated PBMCs from recovered SARS patients over 1 year after recovery, suggesting that if SARS-CoV-specific memory CD8$^+$ T cells were indeed present in peripheral blood of recovered SARS patients over 1 year postinfection, they were at a low frequency.

To expand virus-specific CTL clones, freshly isolated PBMCs were challenged in vitro with heat-inactivated SARS-CoV. The generation of SARS-CoV-specific CTLs was tracked chronologically based on HLA-A*0201/SSp-1 tetramer staining and CD8 expression. For all 13 HLA-A*0201$^+$ recovered SARS patients, tetramer-positive CD8$^+$ T cells were dramatically increased in number 48 h following challenge with SARS-CoV, with levels peaking on day 7 of short-term culture (Fig. 1*A*), indicative of a recall CTL response from memory T cells. The frequency of tetramer-positive CD8$^+$ T cells on day 7 reached 4.98 ± 0.77% (n = 13, Table I and Fig. 1*B*). Day 7 inactivated virus-challenged bulk PBMCs were analyzed using the IFN-γ ELISPOT assay. All three epitopes, SSp-1, S1202, and S978, elicited relatively high T cell responses by IFN-γ release, which indicated no statistical significance (Fig. 2*A*).

To assess function of peptide-specific T cells, HLA-A*0201/SSp-1-positive lymphocytes were positively selected by tetramer

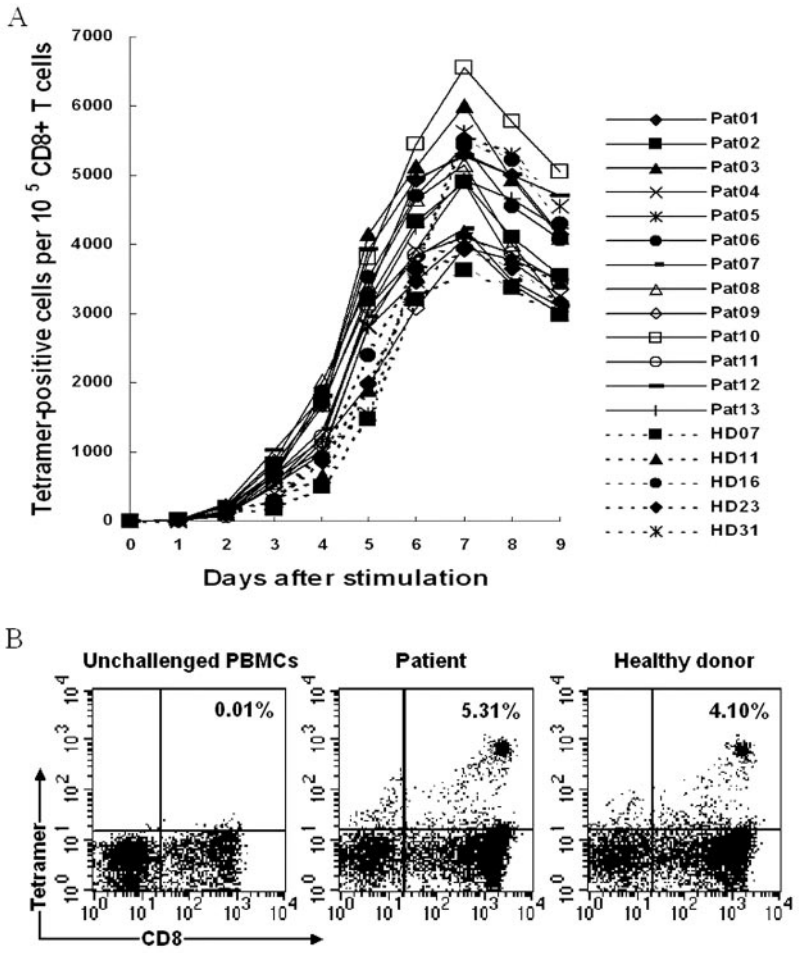

FIGURE 1. CTL response from memory T cells encountering SARS-CoV. *A*, Dynamic curves of tetramer-positive $CD8^+$ T cell expansion. *B*, Day 7 tetramer-positive $CD8^+$ T cells from representative recovered SARS patients and the exceptional healthy donors are shown in the *upper right quadrant*, labeled with the percentage of total $CD8^+$ T cells.

magnetic cell sorting from virus-challenged PBMCs as described in *Materials and Methods*. SSp-1-specific T cells exhibited high functional avidity of Ag recognition, tetramer-positive T cells efficiently and specifically lysing spike protein-expressing HLA-$A2.1^+$ SW480 cells (Fig. 3*A*) but not spike protein-expressing HLA-$A2.1^-$ HT29 cells or β-galactosidase-expressing SW480 and HT29 cells (data not shown). They were also able to efficiently secrete IFN-γ upon stimulation with SSp-1 (Fig. 3*B*), but not in response to HLA-A*0201-restricted irrelevant peptide CAP-1 (data not shown). HLA-A*0201/SSp-1 tetramer-positive $CD8^+$ T cells were not detected in heat-inactivated SARS-CoV-challenged PBMCs from HLA-$A*0201^-$ recovered SARS patients at any time point (data not shown).

CTL response to inactivated SARS-CoV in healthy individuals

We previously investigated in vitro generation of effector CTLs from healthy donors without any contact history with SARS-CoV following repeated challenge of PBMCs with heat-inactivated SARS-CoV-pulsed dendritic cells in culture medium supplemented with IL-7, IL-10, and IL-2. Ag-specific effector CTLs were generated from naive precursors via three or more rounds of weekly repeated challenges in 17 of 36 healthy donors (data not shown). Intriguingly, of the 36 healthy donors, another five healthy donors, donors 7, 11, 16, 23, and 31, composed of three females and two males and aged 47, 38, 51, 42, and 29, respectively, produced a recall-like CTL response from memory T cells upon challenge with heat-inactivated SARS-CoV. HLA-A*0201/SSp-1 tetramer-positive $CD8^+$ T cells were dramatically increased in number 48 h following challenge with SARS-CoV and peaked on day 7, with similar kinetics to those from recovered SARS patients (Fig. 1*A*). On day 7, the frequency of tetramer-positive $CD8^+$ T cells reached $4.31 \pm 0.83\%$ for the 5 exceptional healthy donors, in contrast to $4.98 \pm 0.77\%$ for the 13 recovered SARS patients ($p > 0.05$, Table I and Fig. 1*B*).

Day 7 inactivated virus-challenged bulk PBMCs from the five exceptional healthy donors were analyzed using the IFN-γ ELISPOT assay. SSp-1 elicited a T cell response with a relatively low number of IFN-γ SFC of $241 \pm 67/10^5$ day 7 cultured PBMCs in comparison with $452 \pm 96/10^5$ day 7 cultured PBMCs from recovered SARS patients, but epitope S1202 or S978 did not elicit T cell responses in any of the five exceptional healthy donors. To elucidate the question from which primary infection arose these memory T cell populations cross-reactive to SSp-1 found in the five exceptional healthy donors, we conducted an extensive search for the alternative epitopes recognized by the cross-reactive memory T cell populations using epitope panel including peptides from HCoV-229E spike protein (H77, LLLNCLWSV, residues 77–85; H881, LITGRLAAL, residues 881–889), and dominant epitopes from human influenza A virus matrix protein (GILGFVFTL, residues 58–66), human CMV pp65 (NLVPMVATV, residues 495–503), EBV LMP2 (CLGGLLTMV, residues 426–434), and hepatitis B virus core Ag (FLPSDFFPSV, residues 18–27). Unfortunately, there was no response to these epitopes in the IFN-γ ELISPOT assay for the five exceptional healthy donors (Fig. 2*B*). All of these data suggest that cross-reactive memory T cells to SARS-CoV epitope SSp-1, not to S1202 or S978, may exist in T cell repertoire of a small subset of healthy individuals. However, in this study, we obtained no knowledge of the origin of

Table I. *Characteristics of CD8$^+$ T cells expanded from heat-inactivated SARS-CoV-challenged PBMCs in recovered SARS patients and the exceptional healthy donors*[a]

Donor	Frequency (%) of Tetramer$^+$CD8$^+$ T Cells[b]*	Intracellular Cytokine[c]				Phenotype[d]				
		IFN-γ**	TNF-α**	Perforin**	Granzyme A**	CD45RA	CCR7	CD62L	CCR5	CD44
Patient	4.98 ± 0.77	83.8 ± 3.4	76.3 ± 2.3	79.5 ± 2.9	78.2 ±2.8	+	−	−	+	+
Healthy donor	4.31 ± 0.83	54.9 ± 5.3	53.8 ± 3.7	60.6 ± 2.5	59.1 ± 3.3	+	−	−	+	+

[a] The data from patient donors ($n = 13$) and the exceptional healthy donors ($n = 5$) are presented as the mean ± SD.
[b] The frequency of tetramer-positive CD8$^+$ T cells is shown as the percentage of the total CD8$^+$ T cells upon challenge with heat-inactivated SARS-CoV at day 7.
[c] The frequency of intracellular cytokine-positive CD8$^+$ T cells is shown as the percentage of total day 7 virus-challenged CD8$^+$ T cells.
[d] Phenotypes of day 7 tetramer-positive CD8$^+$ T cells: +, positive, −, negative.
*, $p > 0.05$; **, $p < 0.05$.

these cross-reactive memory T cell populations. Therefore, we focus on differences in properties of SSp-1-specific T cells among the five exceptional healthy donors and the recovered SARS patients.

Although tetramer-positive CD8$^+$ T cells were expanded from PBMCs of the five exceptional healthy donors with similar kinetics to those from recovered SARS patients, they displayed functional impairment. Cytotoxic activity and IFN-γ production of tetramer positively selected T cells from the five exceptional healthy donors were less than one-half of the average displayed by tetramer-positive T cells from recovered SARS patients at all E:T ratios and peptide concentrations tested (Fig. 3).

Intracellular cytokines in virus-challenged PBMCs and phenotypes of tetramer-positive T cells

SSp-1-specific CTLs derived from the exceptional healthy donors displayed impaired cytotoxic activity and low level of IFN-γ production in comparison with those of CTLs from recovered SARS patients; this correlated well with the observation that less intracellular IFN-γ, TNF-α, perforin, and granzyme A accumulated in the exceptional healthy donor-derived CTLs than in CTLs from recovered SARS patients following short-term in vitro expansion. The frequency of intracellular cytokine-positive CD8$^+$ T cells of total day 7 virus-challenged CD8$^+$ T cells in the five exceptional healthy donors was 54.9 ± 5.3% for IFN-γ, 53.8 ± 3.7% for TNF-α, 60.6 ± 2.5% for perforin, and 59.1 ± 3.3% for granzyme A, in contrast to 83.8 ± 3.4% for IFN-γ ($p < 0.05$), 76.3 ± 2.3% for TNF-α ($p < 0.05$), 79.5 ± 2.9% for perforin ($p < 0.05$), and 78.2 ± 2.8% for granzyme A ($p < 0.05$) in the 13 recovered SARS patients (Table I and Fig. 4).

The tetramer-positive T cells in vitro expanded from memory T cells of the 13 recovered SARS patients and the 5 exceptional healthy donors all shared a differentiated effector CTL phenotype, CD45RA$^+$CCR7$^-$CD62L$^-$. They also expressed CCR5 and CD44 (Table I and Fig. 5), molecules indicative of preferential

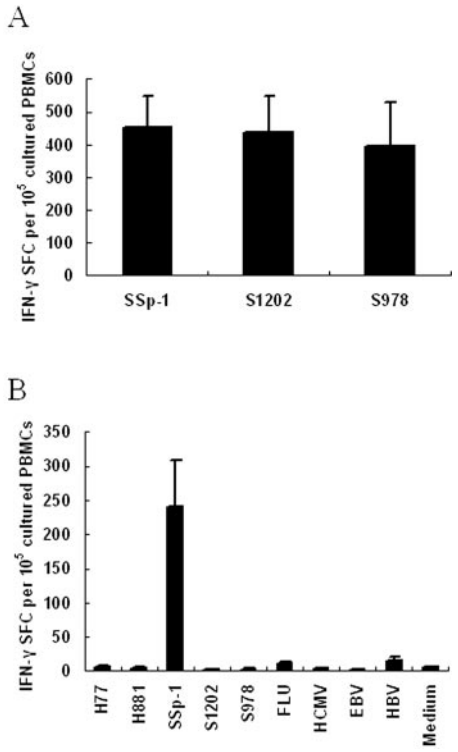

FIGURE 2. Assessment of CTL responses in heat-inactivated SARS-CoV-challenged PBMCs to different epitopes. *A*, SSp-1, S1202, and S978 elicited relatively high T cell responses by IFN-γ release in recovered SARS patients, but the results indicated no statistical significance, $p > 0.05$. *B*, Only SSp-1 but not other epitopes from human coronaviruses or dominant epitopes from other human viruses elicited IFN-γ release in the five exceptional healthy donors.

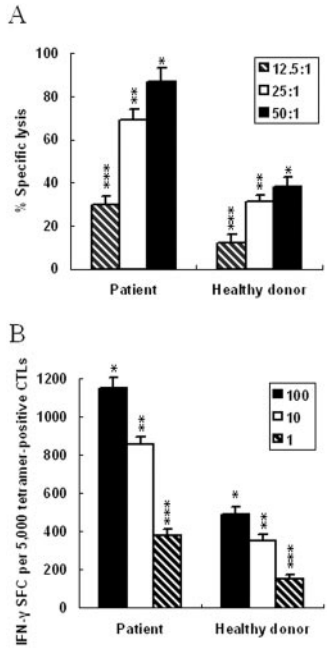

FIGURE 3. Impaired function of CTLs from the exceptional healthy donors compared with those from recovered SARS patients. *A*, Pooled data (healthy donors, $n = 5$; recovered patients, $n = 13$) showing day 7 tetramer-positive CTL lysis of spike protein-expressing HLA-A2.1$^+$ colorectal carcinoma SW480 cells at indicated E:T ratios, as assessed by ^{51}Cr release assays. *B*, Pooled data (healthy donors, $n = 5$; recovered patients, $n = 13$) showing IFN-γ production of day 7 tetramer-positive T cells in response to SSp-1 at indicated peptide concentrations (micromolar), as assessed by ELISPOT. Bars depict mean ± SD. *, **, and ***, $p < 0.01$.

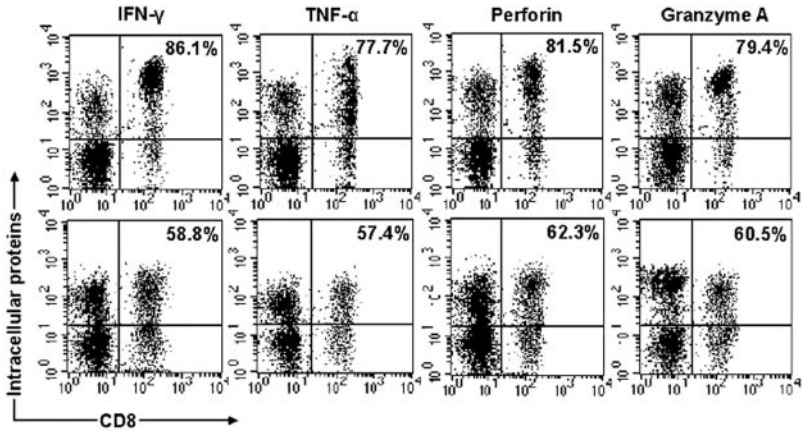

FIGURE 4. Intracellular cytokine expression in virus-challenged PBMCs. Intracellular IFN-γ-, TNF-α-, perforin-, or granzyme A-positive $CD8^+$ T cells from representative recovered SARS patients (*top four panels*) and the exceptional healthy donors (*bottom four panels*) are shown in the *upper right quadrant*, labeled with the percentage of total day 7 virus-challenged $CD8^+$ T cells.

homing to peripheral tissues but not to lymph nodes. However, whether this phenotype might reflect the actual in vivo state of the peptide-specific T cells should be further identified.

Comparison of low-avidity CTLs from the exceptional healthy donors with high-avidity CTLs from recovered SARS patients

To gain further insight into the differences in response magnitude and functional avidity of SSp-1-specific CTLs between recovered SARS patients and the exceptional healthy donors, tetramer-selected CTLs were cloned and maintained from five random selected patient donors and the five exceptional healthy donors as described in *Materials and Methods*. After 1 wk, functional avidity of Ag recognition was determined by ^{51}Cr release assay and IFN-γ ELISPOT assay, assessing the ability of CTL clones to specifically lyse SSp-1-pulsed T2 cells and secrete IFN-γ in the presence of serial dilutions of SSp-1, respectively. The SSp-1 concentration required for half-maximal lysis (EC_{50}) using cloned CTLs as effector cells and SSp-1-pulsed T2 cells as target cells at an E:T ratio of 25:1 varied among the different CTL clones. All CTL clones derived from recovered patients recognized SSp-1 with very high functional avidity with EC_{50} of $(1.34 \pm 0.37) \times 10^{-8}$ peptide M. In contrast, CTL clones from the five exceptional healthy donors exhibited lower avidity Ag recognition with EC_{50} of $(3.18 \pm 1.42) \times 10^{-6}$ peptide M ($p < 0.001$). In correlation with cytotoxic activity, CTL clones derived from the exceptional healthy donors produced equal levels of IFN-γ to those from recovered patients when stimulated with a >2 logs higher peptide dose. The concentration of SSp-1 required to generate 400 SFC per 5000 cloned CTLs was $(0.97 \pm 0.28) \times 10^{-8}$ peptide M for the recovered SARS patients in contrast with $(2.15 \pm 0.49) \times 10^{-6}$ peptide M ($p < 0.001$) for the five exceptional healthy donors (Table II). Differences in functional avidity of Ag recognition of SSp-1-specific CTL clones between recovered SARS patients and the exceptional healthy donors were also revealed in peptide titration curves (Fig. 6, *A* and *B*).

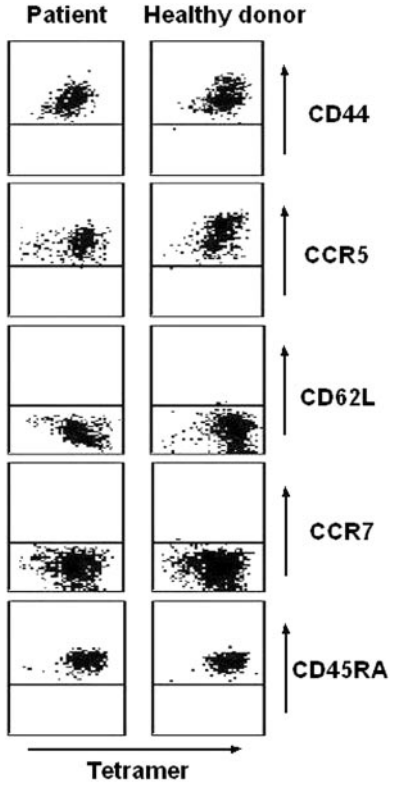

FIGURE 5. Phenotypes of tetramer-positive T cells. Dot plots show $CD45RA^+$, $CCR7^-$, $CD62L^-$, $CCR5^+$, or $CD44^+$ cells in the gated tetramer-positive T cells from representative recovered SARS patients or the exceptional healthy donors.

To further investigate differences in structural avidity between CTL clones derived from recovered SARS patients and the exceptional healthy donors, we performed tetramer dissociation assays as previously described (20–22). Cloned CTLs were incubated with saturating concentrations of PE-labeled HLA-A*0201/SSp-1 tetramers at 4°C, and then washed and incubated with an excess of unlabeled tetramers, and the rate of PE-labeled tetramer dissociation was assayed by flow cytometry. The tetramer dissociated faster from CTL clones derived from the five exceptional healthy donors than from those derived from recovered SARS patients (Fig. 6*C*). The half-time of dissociation of PE-labeled tetrameric complexes from CTL clones as assessed by 50% reduction in fluorescence was 64 ± 6 min for the recovered SARS patients in contrast with 34 ± 3 min ($p < 0.01$) for the five exceptional healthy donors (Table II).

Discussion

Evidence shows that $CD8^+$ CTLs play a pivotal role in both virus elimination and/or pathogenesis of acute respiratory virus infections (23–25). In this study, we first analyzed CTL responses to SARS-CoV in recovered SARS patients. Inactivated SARS-CoV elicited an Ag-specific recall CTL response in PBMCs of recovered SARS patients over 1 year after recovery, suggesting that these individuals generated protective cell-mediated immunity against SARS-CoV following infection with SARS-CoV and developed a self-limiting illness. In contrast, those who suffered from

Table II. *Functional avidity of SSp-1-specific CTL clones derived from recovered SARS patients and the exceptional healthy individuals*[a]

Donor	EC_{50} (Peptide M)[b]*	IFN-γ SFC400 (Peptide M)[c]*	$t_{1/2}$ (min)[d]**
Patient	$(1.34 \pm 0.37) \times 10^{-8}$	$(0.97 \pm 0.28) \times 10^{-8}$	64 ± 6
Healthy donor	$(3.18 \pm 1.42) \times 10^{-6}$	$(2.15 \pm 0.49) \times 10^{-6}$	34 ± 3

[a] All cloned CTLs from patient donors ($n = 5$) and the exceptional healthy donors ($n = 5$) were tested for function after 1 wk of culture. The data are presented as the mean ± SD.
[b] EC_{50} corresponds to the peptide concentration required for half-maximal lysis. Percent specific lysis was obtained using cloned CTLs as effector cells and SSp-1-pulsed T2 cells as target cells, at an E:T ratio of 25:1.
[c] IFN-γ SFC400, defined as the concentration of SSp-1 required to generate 400 SFC per 5000 cloned CTLs.
[d] $t_{1/2}$ corresponds to the half-time of dissociation of PE-labeled tetrameric complexes from CTL clones, as assessed by 50% reduction in fluorescence.
*, $p < 0.001$; **, $p < 0.01$.

critical SARS or died of SARS apparently could not generate sufficient protective immunity to eliminate SARS-CoV; their immune responses to this pathogen may have in fact exacerbated their illness.

We also investigated Ag-specific CTL responses to inactivated SARS-CoV in healthy individuals. Interestingly, 5 of the 36 healthy donors produced a recall-like Ag-specific CTL response upon short-term challenge with heat-inactivated SARS-CoV. Although in vitro expansion of Ag-specific T cells via long-term restimulation would result in substantial functional differences from the original in vivo state of T cells (26), we found that short-term in vitro expansion (not more than 2 wk) did not generate specific T cells from naive precursors (data not shown), consistent with previous findings (27–29). We thus consider that the CTL responses arising in PBMCs of recovered SARS patients and the five exceptional healthy donors 48 h following in vitro culture reflect memory T cells encountering SARS-CoV Ags.

Memory T cells can be divided into two functionally distinct subsets based on expression of chemokine receptor CCR7. CCR7$^-$ memory cells express receptors for migration to inflamed tissues, but CCR7$^+$ memory cells express lymph-node homing receptors (30, 31). The tetramer-positive CTLs activated from memory T cells of both recovered SARS patients and the five exceptional healthy donors exhibited a differentiated effector CTL phenotype, CD45RA$^+$CCR7$^-$CD62L$^-$, and displayed immediate effector function. These cells also expressed CCR5 and CD44, required for preferential homing to peripheral tissues but not to lymph nodes. Correlatively, autopsy of spleen and lymph nodes of patients who died of SARS revealed lymphoid depletion and reduced numbers of lymphocytes (32–34).

These data may reflect CTLs playing dual roles in control of virus replication and immunopathology of acute SARS-CoV infection. SARS-CoV infection of pulmonary tissues causes cells present in these tissues, such as epithelial cells, pneumocytes, matrix cells, and infiltrating APCs, to release cytokines or chemokines that attract virus-specific CCR5$^+$CD44$^+$ CTLs into the lungs, favoring the eradication of virus in self-limiting patients. Concurrent recruitment of CCR5$^+$CD44$^+$ CTLs to peripheral tissues leads to a notable drop of CD8$^+$ T cells in peripheral blood, thus partially contributing to SARS-associated lymphopenia. In contrast, CTLs can also lyse virus-infected cells, and an extreme CTL response may result in tissue injury and the attraction of greater numbers of neutrophils and macrophages to the injured area, as was observed in the alveoli and the interstitium of lung tissues during autopsy of SARS victims (2, 8). This, in turn, induces enhanced production of proinflammatory cytokines from sensitized lymphocytes, monocytes/macrophages, and epithelial cells within the lungs, resulting in cytokine network dysregulation (35), and increases the killing activity of phagocytes and NK cells, thus exacerbating disease. Future studies in acute SARS patients will be essential to find concrete evidence regarding CTL responses to SARS-CoV infection; however, such cases are now rare, due to the decline in the SARS epidemic.

It has been widely known that some cross-reactive memory T cells can be reactivated by heterologous viral infections (36, 37) and that the response to a secondary challenge may be dominated

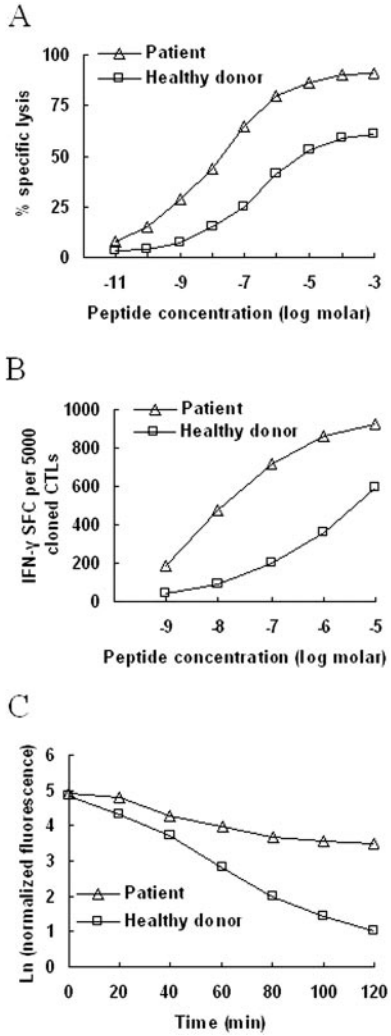

FIGURE 6. Low-avidity SSp-1-specific CTL clones obtained from the exceptional healthy donors compared with high-avidity ones from recovered SARS patients. *A*, Representative data showing cytotoxic activity assessed by ^{51}Cr release assays. *B*, Representative data showing IFN-γ production determined by ELISPOT. *C*, Dissociation kinetics of the interaction between HLA-A*0201/SSp-1 tetramers and cloned CTLs. Dissociation of PE-labeled tetramers from CTLs corresponds to a reduction in fluorescence; low-avidity interactions result in a rapid reduction, whereas CTLs binding tetramers with high avidity maintain fluorescence.

by the expansion of cross-reactive memory T cells induced by the primary infection, which may have lower affinity for the secondary challenging Ag (38–40). This presents the risk that T cell clones activated from cross-reactive memory T cells may have functional impairment, delaying elimination of the secondary challenge virus, and indeed promoting immunopathology to the hosts (41, 42). The lung is the initial site of some virus replication. Reactivation of memory $CD8^+$ T cells in the lung by heterologous agents may cause local immunopathological damage (43). Our results showed that, although PBMCs from the five exceptional healthy donors generated a recall-like Ag-specific T cell response upon challenge with heat-inactivated SARS-CoV, the produced CTLs were of low avidity compared with the high-avidity CTLs derived from memory T cells of recovered SARS patients. Given that the five exceptional healthy donors had no contact history with the newly defined SARS-CoV, one possible explanation for their generation of low-avidity SARS-CoV-specific CTLs may have been the existence of cross-reactive memory T cell clones in their T cell repertoires.

It would be interesting to determine which kinds of pathogens may be responsible for the primary infections that generate memory T cell clones cross-reactive to SARS-CoV epitope SSp-1 but not S978 or S1202; this could provide a means of identifying individuals particularly susceptible to developing critical disease upon subsequent SARS-CoV infection. We extensively evaluated CTL responses to peptides from HCoV-229E and dominant epitopes from human influenza A virus, human CMV, EBV, and hepatitis B virus in the five exceptional healthy donors, but there was no response to these epitopes, and we do not find a homologous peptide to SSp-1 by online database analysis. How a host responds to an infectious agent is a function of its history of previous infections and their influence on the memory T cell pool (41). Unfortunately, current methods for detecting viral-infection history are not optimal, and in many cases, it is impossible to identify the previous infecting pathogens. SARS in children appears to manifest as a milder form of the disease compared with adults (44, 45). Could such a difference be due to immunopathology occurring as a consequence of the reactivation of memory cells, which may be more diverse and prominent in more immunologically mature individuals? To the best of our knowledge, our study is the first report that cross-reactive memory T cells to SARS-CoV may exist in the T cell repertoire of a small subset of healthy individuals.

Considering the severity of the SARS epidemic, there is currently much interest in the generation of SARS vaccines (46–51). A better understanding of the pathogenesis of SARS would greatly help in the development of safe vaccines and therapeutic approaches.

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Disclosures

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References

1. Lee, N., D. Hui, A. Wu, P. Chan, P. Cameron, G. M. Joynt, A. Ahuja, M. Y. Yung, C. B. Leung, K. F. To, et al. 2003. A major outbreak of severe acute respiratory syndrome in Hong Kong. *N. Engl. J. Med.* 348: 1986–1994.
2. Ksiazek, T. G., D. Erdman, C. S. Goldsmith, S. R. Zaki, T. Peret, S. Emery, S. Tong, C. Urbani, J. A. Comer, W. Lim, et al. 2003. A novel coronavirus associated with severe acute respiratory syndrome. *N. Engl. J. Med.* 348: 1953–1966.
3. Peiris, J. S., S. T. Lai, L. L. Poon, Y. Guan, L. Y. Yam, W. Lim, J. Nicholls, W. K. Yee, W. W. Yan, M. T. Cheung, et al. 2003. Coronavirus as a possible cause of severe acute respiratory syndrome. *Lancet* 361: 1319–1325.
4. Drosten, C., S. Gunther, W. Preiser, S. van der Werf, H. R. Brodt, S. Becker, H. Rabenau, M. Panning, L. Kolesnikova, R. A. Fouchier, et al. 2003. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *N. Engl. J. Med.* 348: 1967–1976.
5. Rota, P. A., M. S. Oberste, S. S. Monroe, W. A. Nix, R. Campagnoli, J. P. Icenogle, S. Penaranda, B. Bankamp, K. Maher, M. H. Chen, et al. 2003. Characterization of a novel coronavirus associated with severe acute respiratory syndrome. *Science* 300: 1394–1399.
6. Marra, M. A., S. J. Jones, C. R. Astell, R. A. Holt, A. Brooks-Wilson, Y. S. Butterfield, J. Khattra, J. K. Asano, S. A. Barber, S. Y. Chan, et al. 2003. The genome sequence of the SARS-associated coronavirus. *Science* 300: 1399–1404.
7. Qin, E., Q. Zhu, M. Yu, B. Fan, G. Chang, B. Si, B. Yang, W. Peng, T. Jiang, B. Liu, et al. 2003. A complete sequence and comparative analysis of a SARS-associated virus (Isolate BJ01). *Chin. Sci. Bull.* 48: 941–948.
8. Peiris, J. S., C. M. Chu, V. C. Cheng, K. S. Chan, I. F. Hung, L. L. Poon, K. I. Law, B. S. Tang, T. Y. Hon, C. S. Chan, et al. 2003. Clinical progression and viral load in a community outbreak of coronavirus-associated SARS pneumonia: a prospective study. *Lancet* 361: 1767–1772.
9. Nicholls, J. M., L. L. Poon, K. C. Lee, W. F. Ng, S. T. Lai, C. Y. Leung, C. M. Chu, P. K. Hui, K. L. Mak, W. Lim, et al. 2003. Lung pathology of fatal severe acute respiratory syndrome. *Lancet* 361: 1773–1778.
10. Panesar, N. S. 2003. Lymphopenia in SARS. *Lancet.* 361: 1985.
11. Li, T., Z. Qiu, L. Zhang, Y. Han, W. He, Z. Liu, X. Ma, H. Fan, W. Lu, J. Xie, et al. 2004. Significant changes of peripheral T lymphocyte subsets in patients with severe acute respiratory syndrome. *J. Infect. Dis.* 189: 648–651.
12. Cui, W., Y. Fan, W. Wu, F. Zhang, J. Y. Wang, and A. P. Ni. 2003. Expression of lymphocytes and lymphocyte subsets in patients with severe acute respiratory syndrome. *Clin. Infect. Dis.* 37: 857–859.
13. Wang, B., H. Chen, X. Jiang, M. Zhang, T. Wan, N. Li, X. Zhou, Y. Wu, F. Yang, Y. Yu, et al. 2004. Identification of an HLA-A*0201-restricted $CD8^+$ T-cell epitope SSp-1 of SARS-CoV spike protein. *Blood* 104: 200–206.
14. Wang, Y. D., W. Y. Sin, G. B. Xu, H. H. Yang, T. Y. Wong, X. W. Pang, X. Y. He, H. G. Zhang, J. N. Ng, C. S. Cheng, et al. 2004. T-cell epitopes in severe acute respiratory syndrome (SARS) coronavirus spike protein elicit a specific T-cell immune response in patients who recover from SARS. *J. Virol.* 78: 5612–5618.
15. Wang, Y. D., and W. F. Chen. 2004. Detecting specific cytotoxic T lymphocytes against SARS-coronavirus with DimerX HLA-A2:Ig fusion protein. *Clin. Immunol.* 113: 151–154.
16. Fleischhauer, K., E. Zino, B. Mazzi, G. M. Severini, E. Benazzi, and C. Bordignon. 1996. HLA-A*02 subtype distribution in Caucasians from northern Italy: identification of A*0220. *Tissue Antigens* 48: 673–679.
17. Du Pasquier, R. A., M. J. Kuroda, J. E. Schmitz, Y. Zheng, K. Martin, F. W. Peyerl, M. Lifton, D. Gorgone, P. Autissier, N. L. Letvin, and I. J. Koralnik. 2003. Low frequency of cytotoxic T lymphocytes against the novel HLA-A*0201-restricted JC virus epitope VP1p36 in patients with proven or possible progressive multifocal leukoencephalopathy. *J. Virol.* 77: 11918–11926.
18. Day, C. L., N. P. Seth, M. Lucas, H. Appel, L. Gauthier, G. M. Lauer, G. K. Robbins, Z. M. Szczepiorkowski, D. R. Casson, R. T. Chung, et al. 2003. Ex vivo analysis of human memory CD4 T cells specific for hepatitis C virus using MHC class II tetramers. *J. Clin. Invest.* 112: 831–842.
19. Passoni, L., A. Scardino, C. Bertazzoli, B. Gallo, A. M. Coluccia, F. A. Lemonnier, K. Kosmatopoulos, and C. Gambacorti-Passerini. 2002. ALK as a novel lymphoma-associated tumor antigen: identification of 2 HLA-A2.1-restricted $CD8^+$ T-cell epitopes. *Blood* 99: 2100–2106.
20. Mongkolsapaya, J., W. Dejnirattisai, X. N. Xu, S. Vasanawathana, N. Tangthawornchaikul, A. Chairunsri, S. Sawasdivorn, T. Duangchinda, T. Dong, S. Rowland-Jones, et al. 2003. Original antigenic sin and apoptosis in the pathogenesis of dengue hemorrhagic fever. *Nat. Med.* 9: 921–927.
21. Savage, P. A., J. J. Boniface, and M. M. Davis. 1999. A kinetic basis for T cell receptor repertoire selection during an immune response. *Immunity* 10: 485–492.
22. Davenport, M. P., C. Fazou, A. J. McMichael, and M. F. Callan. 2002. Clonal selection, clonal senescence, and clonal succession: the evolution of the T cell response to infection with a persistent virus. *J. Immunol.* 168: 3309–3317.
23. Hou, S., L. Hyland, K. W. Ryan, A. Portner, and P. C. Doherty. 1994. Virus-specific $CD8^+$ T-cell memory determined by clonal burst size. *Nature* 369: 652–654.
24. Flynn, K. J., G. T. Belz, J. D. Altman, R. Ahmed, D. L. Woodland, and P. C. Doherty. 1998. Virus-specific $CD8^+$ T cells in primary and secondary influenza pneumonia. *Immunity* 8: 683–691.
25. Lukacher, A. E., V. L. Braciale, and T. J. Braciale. 1984. In vivo effector function of influenza virus-specific cytotoxic T lymphocyte clones is highly specific. *J. Exp. Med.* 160: 814–826.
26. Monsurro, V., D. Nagorsen, E. Wang, M. Provenzano, M. E. Dudley, S. A. Rosenberg, and F. M. Marincola. 2002. Functional heterogeneity of vaccine-induced $CD8^+$ T cells. *J. Immunol.* 168: 5933–5942.
27. Janeway, C. A., and K. Bottomly. 1994. Signals and signs for lymphocytes responses. *Cell* 76: 275–285.
28. Celluzzi, C. M., J. I. Mayordomo, W. J. Storkus, M. T. Lotze, and L. D. Falo. 1996. Peptide-pulsed dendritic cells induce antigen-specific, CTL-mediated protective tumor immunity. *J. Exp. Med.* 183: 283–287.

29. Subklewe, M., A. Chahroudi, A. Schmaljohn, M. G. Kurilla, N. Bhardwaj, and R. M. Steinman. 1999. Induction of Epstein-Barr virus-specific cytotoxic T-lymphocyte responses using dendritic cells pulsed with EBNA-3A peptides or UV-inactivated, recombinant EBNA-3A vaccinia virus. *Blood* 94: 1372–1381.
30. Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401: 708–712.
31. Champagne, P., G. S. Ogg, A. S. King, C. Knabenhans, K. Ellefsen, M. Nobile, V. Appay, G. P. Rizzardi, S. Fleury, M. Lipp, et al. 2001. Skewed maturation of memory HIV-specific CD8 T lymphocytes. *Nature* 410: 106–111.
32. Ding, Y., H. Wang, H. Shen, Z. Li, J. Geng, H. Han, J. Cai, X. Li, W. Kang, D. Weng, et al. 2003. The clinical pathology of severe acute respiratory syndrome (SARS): a report from China. *J. Pathol.* 200: 282–289.
33. Lang, Z., L. Zhang, S. Zhang, X. Meng, J. Li, C. Song, L. Sun, and Y. Zhou. 2003. Pathological study on severe acute respiratory syndrome. *Chin. Med. J.* 116: 976–980.
34. Lang, Z. W., L. J. Zhang, S. J. Zhang, X. Meng, J. Q. Li, C. Z. Song, L. Sun, Y. S. Zhou, and D. E. Dwyer. 2003. A clinicopathological study of three cases of severe acute respiratory syndrome (SARS). *Pathology* 35: 526–531.
35. Lee, C. H., R. F. Chen, J. W. Liu, W. T. Yeh, J. C. Chang, P. M. Liu, H. L. Eng, M. C. Lin, and K. D. Yang. 2004. Altered p38 mitogen-activated protein kinase expression in different leukocytes with increment of immunosuppressive mediators in patients with severe acute respiratory syndrome. *J. Immunol.* 172: 7841–7847.
36. Yang, H. Y., P. L. Dundon, S. R. Nahill, and R. M. Welsh. 1989. Virus-induced polyclonal cytotoxic T lymphocyte stimulation. *J. Immunol.* 142: 1710–1718.
37. Selin, L. K., S. R. Nahill, and R. M. Welsh. 1994. Cross-reactivities in memory cytotoxic T lymphocyte recognition of heterologous viruses. *J. Exp. Med.* 179: 1933–1943.
38. Klenerman, P., and R. M. Zinkernagel. 1998. Original antigenic sin impairs cytotoxic T lymphocyte responses to viruses bearing variant epitopes. *Nature* 394: 482–485.
39. Martinez, F. D., A. L. Wright, L. M. Taussig, C. J. Holberg, M. Halonen, and W. J. Morgan. 1995. Asthma and wheezing in the first six years of life. *N. Engl. J. Med.* 332: 133–138.
40. Crowe, J. E. 2001. Respiratory syncytial virus vaccine development. *Vaccine* 20(Suppl. 1): S32–S37.
41. Selin, L. K., S. M. Varga, I. C. Wong, and R. M. Welsh. 1998. Protective heterologous antiviral immunity and enhanced immunopathogenesis mediated by memory T cell populations. *J. Exp. Med.* 188: 1705–1715.
42. Liu, H., S. Andreansky, G. Diaz, S. J. Turner, D. Wodarz, and P. C. Doherty. 2003. Quantitative analysis of long-term virus-specific CD8$^+$-T-cell memory in mice challenged with unrelated pathogens. *J. Virol.* 77: 7756–7763.
43. Chen, H. D., A. E. Fraire, I. Joris, M. A. Brehm, R. M. Welsh, and L. K. Selin. 2001. Memory CD8$^+$ T cells in heterologous antiviral immunity and immunopathology in the lung. *Nat. Immunol.* 2: 1067–1076.
44. Van Bever, H. P., S. Y. Chng, and D. Y. Goh. 2004. Childhood severe acute respiratory syndrome, coronavirus infections and asthma. *Pediatr. Allergy Immunol.* 15: 206–209.
45. Wong, G. W., A. M. Li, P. C. Ng, and T. F. Fok. 2003. Severe acute respiratory syndrome in children. *Pediatr. Pulmonol.* 36: 261–266.
46. Marshall, E., and M. Enserink. 2004. Caution urged on SARS vaccines. *Science* 303: 944–946.
47. Yang, Z. Y., W. P. Kong, Y. Huang, A. Roberts, B. R. Murphy, K. Subbarao, and G. J. Nabel. 2004. A DNA vaccine induces SARS coronavirus neutralization and protective immunity in mice. *Nature* 428: 561–564.
48. Subbarao, K., J. McAuliffe, L. Vogel, G. Fahle, S. Fischer, K. Tatti, M. Packard, W. J. Shieh, S. Zaki, and B. Murphy. 2004. Prior infection and passive transfer of neutralizing antibody prevent replication of severe acute respiratory syndrome coronavirus in the respiratory tract of mice. *J. Virol.* 78: 3572–3577.
49. Kim, T. W., J. H. Lee, C. F. Hung, S. Peng, R. Roden, M. C. Wang, R. Viscidi, Y. C. Tsai, L. He, P. J. Chen, et al. 2004. Generation and characterization of DNA vaccines targeting the nucleocapsid protein of severe acute respiratory syndrome coronavirus. *J. Virol.* 78: 4638–4645.
50. Gao, W., A. Tamin, A. Soloff, L. D'Aiuto, E. Nwanegbo, P. D. Robbins, W. J. Bellini, S. Barratt-Boyes, and A. Gambotto. 2003. Effects of a SARS-associated coronavirus vaccine in monkeys. *Lancet* 362: 1895–1896.
51. Bisht, H., A. Roberts, L. Vogel, A. Bukreyev, P. L. Collins, B. R. Murphy, K. Subbarao, and B. Moss. 2004. Severe acute respiratory syndrome coronavirus spike protein expressed by attenuated vaccinia virus protectively immunizes mice. *Proc. Natl. Acad. Sci. USA* 101: 6641–6646.