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Human memory T cell responses to SARS-CoV E protein

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Received 20 March 2006; accepted 15 May 2006
Available online 30 June 2006

Abstract

E protein is a membrane component of severe acute respiratory syndrome coronavirus (SARS-CoV). Disruption of E protein may reduce viral infectivity. Thus, the SARS-CoV E protein is considered a potential target for the development of antiviral drugs. However, the cellular immune responses to E protein remain unclear in humans. In this study, we found that peripheral blood mononuclear cells (PBMCs) from fully recovered SARS individuals rapidly produced IFN-γ and IL-2 following stimulation with a pool of 9 peptides overlapping the entire E protein sequence. Analysis of the immune responses by flow cytometry showed that both CD4+ and CD8+ T cells were involved in the SARS-CoV E-specific immune responses after stimulation with SARS-CoV E peptides. Moreover, the majority of IFN-γ+CD4+ T cells were central memory cells expressing CD45RO+CCR7+CD62L+; whereas IFN-γ+CD8+ memory T cells were mostly effector memory cells expressing CD45RO+CCR7−CD62L−. The results of T-cell responses to 9 individual peptides indicated that the E protein contained at least two major T cell epitopes (E2 amino acid [aa] 9–26 and E5–6: aa 33–57) which were important in eliciting cellular immune response to SARS-CoV E protein in humans.

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Keywords: SARS coronavirus; E protein; Memory T cells; Epitope; Human

1. Introduction

Severe acute respiratory syndrome (SARS) is a newly emerged infectious disease caused by a novel type of coronavirus, designated SARS coronavirus (SARS-CoV) [1–3]. During the period January 2003 to May 2003, SARS spread rapidly among large human populations and led to thousands of infected patients and hundreds of deaths in over 30 countries [4–6]. As reported, substantial efforts have been made to isolate the SARS-CoV and study its genomic sequence [5,6], and its ACE-2 binding receptors have been identified on target cells [7]. Increasing evidence in both animal and human models indicates that SARS-CoV infection results in multiple organ dysfunction [8,9]. Moreover, it has been also reported that SARS-CoV infection induces specific antibody production that is considered both an effective method of diagnosis, as well as potential for a passive and active immunization for the prevention and treatment of SARS [10–12]. In addition to the induction of humoral immune responses, cell-mediated immune responses to SARS-CoV were detected in both SARS patients and animal models [13–15]. Clinical data showed that pronounced lymphopenia was observed in most SARS patients [16]. All of those reports show that T cell responses may not only participate in the clearance of virus in recovered SARS patients but also contribute to immunopathology in early stages of the disease. Therefore, it is very
important to understand the role of T lymphocytes in human SARS-CoV infection.

SARS-CoV has four structural proteins (S, N, M, and E proteins) that have various functions [5,6]. Many reports concerning the immune responses to SARS-CoV S and N proteins have been published [17–19], the immune responses against E protein in human and animal models have not yet been studied extensively [20]. E protein is a small, 9–12 kDa integral membrane protein. A previous study demonstrated that SARS-CoV E protein could form ion channels [21]. E protein also plays a role in viral assembly and morphogenesis [22]. Disruption of the E protein may possibly abrogate viral infectivity [23]. Recently, Yang et al. [24] showed that SARS-CoV E protein induced apoptosis in transfected Jurkat T cells, which possibly contributes to the SARS-CoV-induced lymphopenia observed in most SARS patients. Thus SARS-CoV E protein may be a potential target for the development of antiviral drugs. Here we analyzed memory T cell responses against the E protein in a group of individuals who had clinical SARS-CoV infection between January and May 2003. Our results demonstrated that SARS-CoV E protein might contain at least two different major epitopes, aa 9–26 and aa 33–57, which could be important in eliciting cellular immune response against SARS-CoV E protein in humans.

2. Materials and methods

2.1. Subjects

Eleven recovered SARS individuals (5 males and 6 females, aged 20 to 37) were recruited from the Second Affiliated Hospital of Sun Yat-sen University and Guangdong Provincial Hospital of Traditional Chinese Medicine, Guangzhou, Guangdong, China. All participants had been diagnosed as SARS patients based on clinical examination during the period January to May 2003. The diagnostic criteria for SARS-CoV infection followed the World Health Organization definition of SARS [25]. The diagnosis of SARS-CoV infection was further confirmed by serological detection of SARS-CoV-specific antibodies [26]. Normal subjects without any contact history with SARS patients were used as negative controls.

2.2. SARS-CoV E peptides

Nine synthetic peptides that spanned the entire sequence of the SARS-CoV E protein were kindly provided by Drs. Koup and Bailer at the Vaccine Research Center of the National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), USA. The peptides used in the subsequent experiments were 16–20 mers, overlapped by 10 amino acids and named E1–E9.

2.3. Preparation of PBMCs

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation. Cells were washed twice with RPMI-1640 (GIBCO) and suspended in complete culture medium (RPMI-1640 containing 10% fetal calf serum, 100 U of penicillin per ml and 100 mg of streptomycin per ml).

2.4. Measurement of IFN-γ production in culture supernatant by ELISA

PBMCs were seeded into the wells of 96-well culture plates (Becton Dickinson) (2 × 10^5 cells/well) in triplicate. Peptides and costimulatory mAbs to CD28 (BD Pharmingen) and CD49d (BD Pharmingen) were added, each at 1 µg/ml, to the wells. In addition, PBMCs stimulated with mAbs to CD3 and CD28 were used as a positive control; as a negative control, PBMCs were not stimulated. The plates were incubated for 72 h at 37 °C in a humidified atmosphere containing 5% CO2 and 95% air. Supernatants were collected and the level of IFN-γ was measured by ELISA kit (R&D) according to the manufacture’s instructions. The detection limit of the IFN-γ assay kit was 15 pg/ml.

2.5. Detection of IFN-γ production by ELISPOT assay

ELISPOT assay kit for IFN-γ was purchased from BD Biosciences and assays performed as described [27]. Briefly, 96-well plates (Millipore) were coated with anti-IFN-γ mAb and incubated at 4 °C overnight. The plates were washed three times before blocking with complete culture medium. Fresh PBMCs were plated at 2 × 10^5 cells per well in triplicate. Peptides and costimulatory mAbs to CD28 and CD49d were added at 1 µg/ml each. PBMCs stimulated with CD3 and CD28 (1 µg/ml) mAbs were used as a positive control, and as a negative control, PBMCs were not stimulated. After incubation for 72 h at 37 °C, the cells were removed and incubated with biotinylated anti-human IFN-γ detection antibody for 2 h at room temperature. After washing, wells were developed for 1 h with streptavidin-HRP, followed by incubation with substrate reagent according to the manufacturer’s protocol. Spot-forming cells (SFC) were detected with the ELISPOT image analysis system (Sage Creation). The frequency of IFN-γ-producing cells was calculated as the number of spots/number of total PBMCs per well and was adjusted as the number of IFN-γ-producing cells/10^6 PBMCs. The number of spots in negative control wells was in a range of 0–2 spots.

2.6. Intracellular cytokine staining and flow cytometry analysis

Intracellular cytokines IFN-γ and IL-2 were assessed as previously described [28]. PBMCs (1 × 10^6 cells) were placed into polystyrene tissue culture tubes (Becton Dickinson) with 1 ml of complete medium (CM) and were stimulated with peptides plus costimulatory mAbs as described above. Culture
tubes were incubated at 37°C in a humidified 5% CO₂ atmosphere for 6 h. Brefeldin A (Sigma-Aldrich) was added to cells at a final concentration of 10 μg/ml to prevent secretion of cytokines. After incubation, the cells were harvested and washed twice with PBS containing 0.1% BSA plus 0.05% sodium azide. Cell phenotype was determined by cell surface staining with PerCP-labeled anti-CD4, PerCP-labeled anti-CD8, FITC-labeled anti-CD45RO, PE-labeled anti-CCR7 and PE-labeled anti-CD62L mAbs (BD Pharmingen) at 4°C for 30 min. For intracellular IFN-γ or IL-2 detection, APC-labeled anti-IFN-γ and PE-labeled anti-IL-2 (BD Pharmingen) antibodies were used. Briefly, cells were fixed with 4% PFA for 8 min at room temperature, washed in PBS-BSA buffer and incubated for 1 h with in PBS-BSA buffer containing 0.1% saponin. Cells were washed in PBS-BSA-0.1% saponin buffer and stained with the labeled Abs at 4°C for 30 min. Data acquisition was performed on a flow cytometer (FACS Calibur). The data were analyzed using CellQuest 4.3 software (Becton Dickinson).

3. Results

3.1. Memory T cell response specific for SARS-CoV E antigen two years after recovery

To assess memory T cell response specific for E protein after SARS-CoV infection in humans, PBMCs from individuals who had fully recovered from SARS two years after infection were stimulated with a pool of 9 peptides spanning the entire amino acid sequence of the SARS-CoV E protein, or the cells were stimulated with anti-CD3 and anti-CD28 antibodies under the same culture conditions as positive controls. After incubation for 72 h, the culture supernatants were collected and assessed for the production of IFN-γ by ELISA. As shown in Fig. 1A, stimulation of PBMCs from all SARS-recovered donors with a pool of SARS-CoV peptides resulted in significantly higher levels of IFN-γ production compared with the cells from normal individuals. Similarly, the frequency of SARS-CoV E antigen-specific IFN-γ-producing cells determined by IFN-γ ELISPOT assay in PBMCs from SARS donors with a mean frequency of 0.072% (range: 0.05–0.12%). Together, these data demonstrated that the T cell response specific to E protein could persist for two years after SARS-CoV infection.

3.2. Characterization of memory T cells specific for E protein by flow cytometry

To analyze the cell populations involved in the E protein-specific T cell response, multiple-color flow cytometry was used to characterize the phenotype and determine the frequency of cytokine-producing cells. PBMCs from SARS-recovered donors were incubated with a pool of E peptides and stained for cell surface expression of CD4 and CD8 and for intracellular IFN-γ expression (Fig. 2A). The results showed that IFN-γ-producing CD4+ T cells in response to E protein were detected in all of the SARS-recovered donors with a mean frequency of 0.072% (range: 0.05–0.09%). The frequency of IFN-γ-producing CD8+ T cells specific for E protein was similar to that of the CD4+ T cells (mean: 0.078%, range: 0.05–0.12%). Together, these data demonstrated that both CD4+ and CD8+ T cells were involved in SARS-CoV E-specific immune responses.
In addition to detection of IFN-γ expression, the T cell responses to SARS-CoV E peptides based on their ability to express IL-2 were also evaluated by flow cytometry. PBMCs from SARS-recovered donors were stimulated with a pool of peptides for 6 h. Cell surface and intracellular cytokine staining for IFN-γ was performed. The cells were first gated on CD8⁺ and CD4⁺ T cells and subsequently analyzed for IFN-γ expression. (A) Results shown are from one experiment performed on PBMCs from a SARS patient and are representative of 5 independent experiments from 5 SARS patients. (B) Results represent the expression of IFN-γ as percentage of total CD8⁺ or CD8⁺ T cells in different patients. Bars indicate mean values.

In addition to detection of IFN-γ expression, the T cell responses to SARS-CoV E peptides based on their ability to express IL-2 were also evaluated by flow cytometry. PBMCs from SARS-recovered donors were stimulated with a pool of E peptides and were stained for cell surface expression of CD4 and CD8 and also for intracellular IL-2 expression (Fig. 3). The results showed that SARS-CoV E protein specific CD4⁺ memory T cells were also capable of producing IL-2 (Fig. 3A), and a very low number of specific CD8⁺ memory T cells could produce IL-2 (data not shown). To further analyze the subsets of IL-2-and IFN-γ-producing CD4⁺T cells, co-staining for IFN-γ and IL-2 expression was performed and analyzed by flow cytometry. The results, indicated in Fig. 3B, show that T cells specific for SARS-CoV E peptides could be divided into three subsets based on IL-2 and IFN-γ expression: IFN-γ secreting cells; co-expression of IL-2 and IFN-γ secreting cells; and IL-2 secreting cells. The majority of T cells specific for SARS-CoV E peptides were single IFN-γ-secreting cells, and the frequency of cells secreting both IL-2 and IFN-γ was very low in specific CD4⁺ T cells (Fig. 3C).
The phenotypic characteristics of SARS-CoV E protein specific CD4+ and CD8+ memory T cells were further assessed. Following stimulation ex vivo with E peptides, PBMCs from SARS-recovered donors were stained for surface expression of CD4, CD8, CD45RO, CR7, CD62L and for intracellular IFN-γ and subsequently analyzed by flow cytometry (Fig. 4). The results showed that 75% of IFN-γ+CD4+ T cells were CCR7+, 54.0% of them were CD62L−, and 94% of them expressed CD45RO. Compared to IFN-γ+CD4+ T cells, 70% of IFN-γ+CD8+ T cells did not express CCR7, 97% of them were CD62L−, and 94% of them were CD45RO−. Overall, these data indicated that IFN-γ+CD4+ T cells were mostly contained within the CD45RO−CCR7−CD62L− cell population, whereas most IFN-γ+CD8+ T cells were contained within the CD45RO−CCR7+CD62L− cell population, further suggesting that CD4+ and CD8+ memory T cells are different in phenotype and also demonstrating the heterogeneity of CD4+ and CD8+ memory T cells.

3.3. Identification of immunodominant T cell epitopes in SARS-CoV E protein

PBMCs from SARS-recovered donors were tested in a single peptide screening from 9 overlapping peptides of SARS-CoV E protein by using IFN-γ ELISPOT assay. Although responses were obtained with all nine peptides in one or more donors, the highest responses were obtained with peptides E2 (aa 9 to 26), E5 (aa 33 to 49) and E6 (aa 40 to 57), whereas the rest of the peptides induced lower responses (Fig. 5). Therefore, peptide E2 (aa 9 to 26) and peptides E5, E6 (aa 33 to 57) were the major dominant antigen sites of E protein and contained at least two different epitopes.

3.4. IFN-γ expression by CD4+ and CD8+ T cells in response to individual peptide

Peptides E2, E5 and E6 could effectively stimulate T cell responses when detected with ELISPOT assay. To assess the populations of T cells for the response to individual peptides, PBMCs from SARS-CoV-recovered donors were stimulated for 6 h ex vivo in the presence or absence of peptides E2, E5 or E6. Then the cells were stained for surface CD8 and CD4 and for intracellular IFN-γ and analyzed by flow cytometry (Fig. 6). The frequency of SARS-CoV E-peptide-specific T cells induced by the selected peptides ranged from 0.04 to 0.08% of CD4+ T cells and from 0.02 to 0.08% of CD8+ T cells. These data indicated that the individual peptides E2, E5 and E6 were able to induce the immune responses of both CD4+ and CD8+ memory T cells.

4. Discussion

Our study provides the first evidence that CD4+ and CD8+ memory T-cell responses to SARS-CoV E protein were generated and persistent in the individuals who had had SARS caused by SARS-CoV infection two years previously. In line with our observation, it has recently been shown that immunization of mice with DNA vaccine of the E, M, and N genes can induce high levels of specific antibodies, T cell proliferation, IFN-γ, DTH responses, and in vivo cytotoxic T cell activities in response to SARS-CoV antigens [20]. However, compared with pcD3d/N vaccine, a lower level of immune responses is generated by the pcD3d/E vaccine. In addition, it has been reported that BHPIV-based SARS-CoV DNA vaccines encoding N, M, E or ME protein are not able to generate neutralizing antibody and detectable resistance to SARS-CoV challenge.
while SARS-CoV DNA vaccine encoding S is capable of inducing neutralizing antibody, and its protective efficacy can increase slightly by coexpression of M and E[29]. Therefore, the E protein can induce a lower level of specific immune response compared with other structural proteins of SARS-CoV.

Our results demonstrated that E protein-specific memory T cell response was persistent two years after SARS-CoV infection in humans. In another study by our group, we found that N protein-specific memory T cell responses were readily observed two years after SARS-CoV infection. However, memory T cell responses specific to E protein are much lower, compared to those of N protein (Wu et al., authors’ unpublished data), probably due to its small size and low abundance in the virions, and also possibly due to the SARS-CoV-E protein-induced lymphopenia via apoptosis.

Previously, it was believed that memory T cells could be divided into two functionally distinct subsets based on expression of CCR7[30,31]. CCR7<sup>−</sup> effector memory T cells (TEM cells) were present in the blood, spleen and non-lymphoid tissues, whereas CCR7<sup>+</sup> central memory T cells (TCM cells) were found in lymph nodes, spleen and blood but not in non-lymphoid tissues. Recent studies in both mice and humans have demonstrated that these two cell subsets were able to rapidly produce IFN-γ and TNF-α. The IL-2 production remains a property of TEM cells following stimulation with antigen [32–34]. Our results showed that SARS-CoV-specific IFN-γ<sup>+</sup>CD4<sup>+</sup>T cells were mostly contained within the CD45RO<sup>−</sup>CCR7<sup>−</sup>CD62L<sup>−</sup> cell population, and most IFN-γ<sup>+</sup>CD8<sup>+</sup>T cells were contained within the CD45RO<sup>−</sup>CCR7<sup>−</sup>CD62L<sup>−</sup> cell population. Moreover, these two cell subsets from SARS-recovered donors’ subsets were able to rapidly produce IFN-γ in response to SARS-CoV E peptides. While the frequency of IL-2-secreting CD4<sup>+</sup> memory T cells was much higher than that of CD8<sup>+</sup> memory T cells, consistently with other studies on CD4<sup>+</sup> memory T cells [33], our observations also demonstrated that CD4<sup>+</sup> and CD8<sup>+</sup> memory T cells were different in phenotype and function.

As the T-cell epitopes are usually 8 to 10 aa long, the E peptides used in our study overlapped by 10 aa in each peptide to minimize the possibility of missing the T-cell epitopes of SARS-CoV E protein. The clear difference in protein recognition among SARS-CoV immune donors suggests that the nature and composition of the immune response against the virus varies between individuals possibly due to the difference in HLA types. Although the results showed that the T-cell
epitopes were scattered throughout the sequence of E protein, some peptides were recognized more frequently than others. The peptides that were frequently recognized by T cells were E2 (aa 9 to 26), E5 (aa 33 to 49) and E6 (aa 40 to 57). Therefore, at least two different epitopes, corresponding to aa 9 to 26 and aa 33 to 57, were the major dominant antigen site on E protein for T cell responses. As E protein with 76 amino acids has limited immunogenicity, very few studies have focused on identification of epitopes on E protein. Liu et al. [35] selected specific recombinant scFv antibodies against E and N protein of SARS-CoV from the phage display library and found that the two scFv antibodies B10 and C20 could recognize the non-overlapping epitopes of E protein of SARS-CoV.

In conclusion, the current study demonstrates that both CD4+ and CD8+ T cells participate in SARS-CoV E-specific immune responses and that the memory T cell immune responses specific for SARS-CoV E antigen are persistent for a long period of time after recovery. Peptides E2 (aa 9 to 26), E5 (aa 33 to 49) and E6 (aa 40 to 57) are important epitopes of SARS-CoV E antigen. Persistence of E-specific memory T cell responses in SARS patients may play an important role in protection from SARS-CoV re-infection.

Acknowledgements

This study was supported by grants from National Nature Science Foundation of China (No. 30340012); Scientific Technology Program of Guangdong (No. 2003Z3-E0491); Ministry of Education, Guangdong Province of China and the National Key Basic Research Program of China (No. 2001CB510007). We thank the individuals who donated their blood for this study. We also thank Z. Peng for performing flow cytometry assays.

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