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Longitudinal Analysis of Memory T-Cell Responses in Survivors of Middle East Respiratory Syndrome

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Background. Middle East respiratory syndrome (MERS) is a highly lethal respiratory disease caused by a zoonotic betacoronavirus. The development of effective vaccines and control measures requires a thorough understanding of the immune response to this viral infection.

Methods. We investigated cellular immune responses up to 5 years after infection in a cohort of 59 MERS survivors by performing enzyme-linked immunospot assay and intracellular cytokine staining after stimulation of peripheral blood mononuclear cells with synthetic viral peptides.

Results. Memory T-cell responses were detected in 82%, 75%, 69%, 64%, and 64% of MERS survivors from 1–5 years post-infection, respectively. Although the frequency of virus-specific interferon gamma (IFN-γ)–secreting T cells tended to be higher in moderately/severely ill patients than in mildly ill patients during the early period of follow-up, there was no significant difference among the different clinical severity groups across all time points. While both CD4+ and CD8+ T cells were involved in memory T-cell responses, CD4+ T cells persisted slightly longer than CD8+ T cells. Both memory CD4+ and CD8+ T cells recognized the E/M/N proteins better than the S protein and maintained their polyfunctionality throughout the period examined. Memory T-cell responses correlated positively with antibody responses during the initial 3–4 years but not with maximum viral loads at any time point.

Conclusions. These findings advance our understanding of the dynamics of virus-specific memory T-cell immunity after MERS-coronavirus infection, which is relevant to the development of effective T cell–based vaccines.

Keywords. MERS-CoV; memory T cells; longitudinal analysis.

The Middle East respiratory syndrome coronavirus (MERS-CoV) is one of the newly discovered coronaviruses that can cause fatal pneumonia in humans. While the biological properties of this virus have been relatively well elucidated [1], therapeutic agents and preventive vaccines are not yet available.

A few studies on the immune responses to MERS-CoV infection in human patients have demonstrated that MERS-CoV–specific antibodies, including neutralizing antibodies (nAbs), were generated in most infected persons in proportion to disease severity [2, 3]. While this humoral immune response plays an important role in preventing the spread of viral infection, it cannot stop proceeding to death in fatal infections [2, 3]. In contrast, little information is available on human T-cell responses in MERS-CoV infection. We previously examined the cellular immune response in patients with MERS at the early stage of infection and showed that CD4+ T-cell responses were detected at the convalescent phase of infection in a clinical severity–dependent manner, while CD8+ T-cell responses were observed during the acute stage of infection when CD4+ T-cell responses were not yet detected in some severely ill patients [4]. The latter finding might infer the pathogenic role of CD8+ T cells in the acute phase of infection. However, it has not been resolved whether the presence of memory T-cell responses could prevent infection and/or alleviate clinical symptoms. Furthermore, few studies have reported the longitudinal analysis of virus-specific memory T cells after natural MERS-CoV infection in humans, which is pivotal for the development of effective control measures.

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In the present study, we followed a cohort of patients who recovered from the epidemic infection in 2015 in South Korea for up to 5 years to investigate the magnitude, persistence, and functional features of MERS-CoV–reactive memory T-cell responses after recovery from infection. We also analyzed its relationship with virus-specific antibody responses, viral loads, and clinical severity.

METHODS

Patients

A cohort of 59 patients who recovered from MERS-CoV infection during the 2015 outbreak in Korea participated in this study. These patients were recruited from 5 hospitals in Korea, and this study was approved by the ethical committee of the corresponding hospitals (National Medical Center; H-1510-059-007 and H-1712-085-005, Seoul National University Hospital; 1509-103-705 and 1511-117-723, Seoul Medical Center; 2015-12-102, Dankook University Hospital; 2016-02-014, and Chungnam National University Hospital; 2017-12-004). Their demographic characteristics are presented in Table 1. Samples from uninfected healthy donors were collected either before December 2014 (n = 12) or in April 2018–May 2018 (n = 30). All participants provided written informed consent.

All other experimental methods are available in the Supplementary Materials.

RESULTS

Cohort Characteristics

We enrolled and longitudinally analyzed 59 patients infected with MERS-CoV during the 2015 epidemic outbreak in South Korea. Participants were divided into 3 groups depending on the severity of the illness, as described in our previous study [4]. In brief, the severe group included patients who required mechanical ventilation (n = 17). The moderate group included patients with pneumonia but without respiratory failure (n = 28). The mild group consisted of patients without distinctive pulmonary lesions (n = 14). There was no difference in age and the presence of underlying diseases among the 3 groups, but male sex was dominant in the severe group in our cohort (Table 1). Some patients received antiviral treatment during admission (Table 1), but none received steroid therapy. The maximum viral loads [$\log_{10}$(copy/mL)] during their acute illness were 6.03 (5.19–7.61) (median and range), 7.39 (4.65–9.15), and 8.01 (4.54–9.61) for mild, moderate, and severe groups, respectively.

Kinetics of Memory T-Cell Responses

To evaluate the dynamics of MERS-CoV–specific T lymphocytes in recovered MERS patients, we performed an enzyme-linked immunospot (ELISPOT) assay using peripheral blood mononuclear cells (PBMCs) obtained from different time points after infection. When PBMCs were stimulated with synthetic viral peptides encompassing the 4 structural proteins, interferon gamma (IFN-γ)–producing T cells could be distinctively visualized in the first year after infection, especially in the moderate and severe groups, and these decreased gradually over time in most participants (Figure 1A Supplementary Figures 1 and 2). When all groups were combined, the median frequency of antigen-reactive T cells per 2×10^5 PBMCs at the first, third, and fifth years were 90 (interquartile range [IQR], 49–167), 64 (30–116), and 46 (22–76), respectively (Figure 1B). In a comparative analysis among groups classified per clinical severity, antigen-specific T lymphocytes tended to be observed in higher numbers in the moderate and severe groups than in the mild group at the early time points after infection, although it was not significant. Thus, the median frequency and IQR of IFN-γ–producing T cells observed in the severe, moderate, and mild groups in the first year after infection were 116 (59–258), 96 (60–188), and 44 (34–91), respectively. However, this apparent difference almost disappeared after 3 years of recovery from infection because the number of antigen-reactive T cells decreased more rapidly in the severe/moderate group than in the mild group at this time interval (Figures 1A

Table 1. Characteristics of Patients With Middle East Respiratory Syndrome

Characteristic	Group (Clinical Severity)			Total
	Mild	Moderate	Severe	
No. of patients (%)	14 (23.7)	28 (47.5)	17 (28.8)	59
Male/female, n	6/8	15/13	14/3	35/24
Age, mean ± standard deviation, years	54 ± 16	55 ± 11	53 ± 12	54 ± 12
Person with underlying diseases,[a] n (%)	5 (33.3)	8 (28.6)	6 (35.3)	19 (32.2)
Smoker, n (%)	0 (0)	7 (25.0)	6 (35.3)	13 (22.0)
Person with antiviral treatment,[b] n (%)	6 (42.9)	22 (78.6)	16 (94.1)[c]	44 (74.6)

[a]Diabetes; hypertension; chronic kidney, heart, lung, or liver disease; obesity.

[b]Interferon + ribavirin ± lopinavir/ritonavir.

[c]Three patients also received convalescent serum.

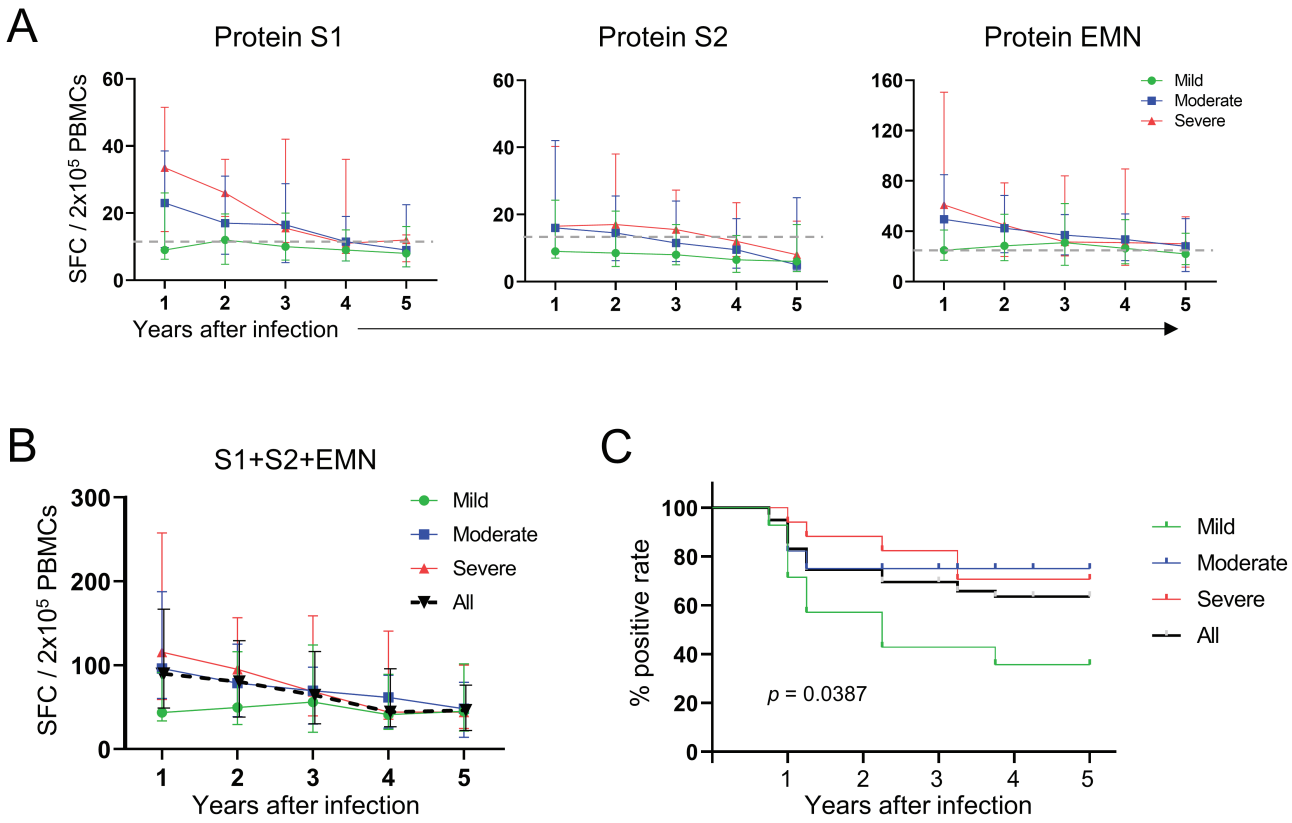

Figure 1. Magnitude and durability of antiviral T-cell responses in Middle East respiratory syndrome (MERS) survivors. Ex vivo interferon-γ enzyme-linked immunospot responses to MERS-coronavirus structural proteins. *A,* The response to each peptide pool. *B,* The combined response to the 3 peptide pools. *C,* The positivity rate of memory T-cell responses in MERS survivors according to the time lapse after infection. The data were analyzed using the log-rank test. The number of participants in the mild, moderate, and severe groups for each year was 6, 20, and 12 for the first year; 14, 28, and 17 for the second year; 11, 24, and 16 for the third year; 10, 22, and 13 for the fourth year; and 9, 17, and 13 for the fifth year. Data A and B represent the median and interquartile range for SFCs per 2×10^5 PBMCs during the 5 years of follow-up. The dashed line in A indicates the cutoff value for a positive response. Abbreviations: PBMC, peripheral blood mononuclear cell; SFC, spot-forming cell.

and B). In terms of the positivity rate of memory T-cell responses, 82%, 69%, and 64% of all participants maintained detectable levels of memory T cells in their peripheral blood in the first, third, and fifth years after infection, respectively (Figure 1C). Importantly, the positivity rate decreased more rapidly in the mild group than in the moderate/severe group ($P < .05$), yielding 36% of mild patients who maintained positive memory T-cell responses in the fifth year after infection, while 70%–74% of moderate/severe patients did (Figure 1C).

Next, we determined the cytokine profile of MERS-specific T cells using intracellular cytokine staining (ICS) following stimulation with MERS-CoV peptide pools. Overall, even if there was no significant difference, a higher frequency of IFN-γ–secreting cells was detected in the CD8+ T cells than in the CD4+ T-cell compartment (0.081%, 0.021–0.1760 vs 0.061%, 0.028–0.108, median and IQR at first year) at the beginning of the follow-up period. However, antiviral CD8+ T cells decreased faster than CD4+ T cells, indicating that the frequency difference between the 2 T-cell subsets gradually decreased and reversed in the last year of observation (Figure 2A Supplementary Figure 3). In comparison among groups, slightly higher frequencies of IFN-γ–producing CD4+ T cells were observed in the moderate/severe group compared with the mild group, while IFN-γ–producing CD8+ T cells were observed at a lower frequency in the severe group than in the other 2 groups (Figure 2A). Based on individual study patients, both CD4+ and CD8+ T lymphocytes contributed to the IFN-γ–secreting T-cell compartment either alone or together (Figure 2B). However, there was no significant correlation between the frequencies of virus-reactive CD4+ and CD8+ T cells across the entire study period (Figure 2C). Although MERS-CoV–reactive CD4+ T-cell responses decreased over time in most patients, they were maintained or even slightly increased in some patients (12 of 56) regardless of the severity of illness (Supplementary Figure 4). A similar pattern of responses was observed in individual patients in the CD8+ T-cell compartment as in CD4+ T cells.

When we analyzed the responsiveness of memory T cells to different viral proteins, it was revealed that irrespective of T-cell subsets, more T cells were responsive to the E/M/N proteins than to the S protein at most time points. This preferential response to the E/M/N proteins was not associated with any specific severity group (Figure 3).

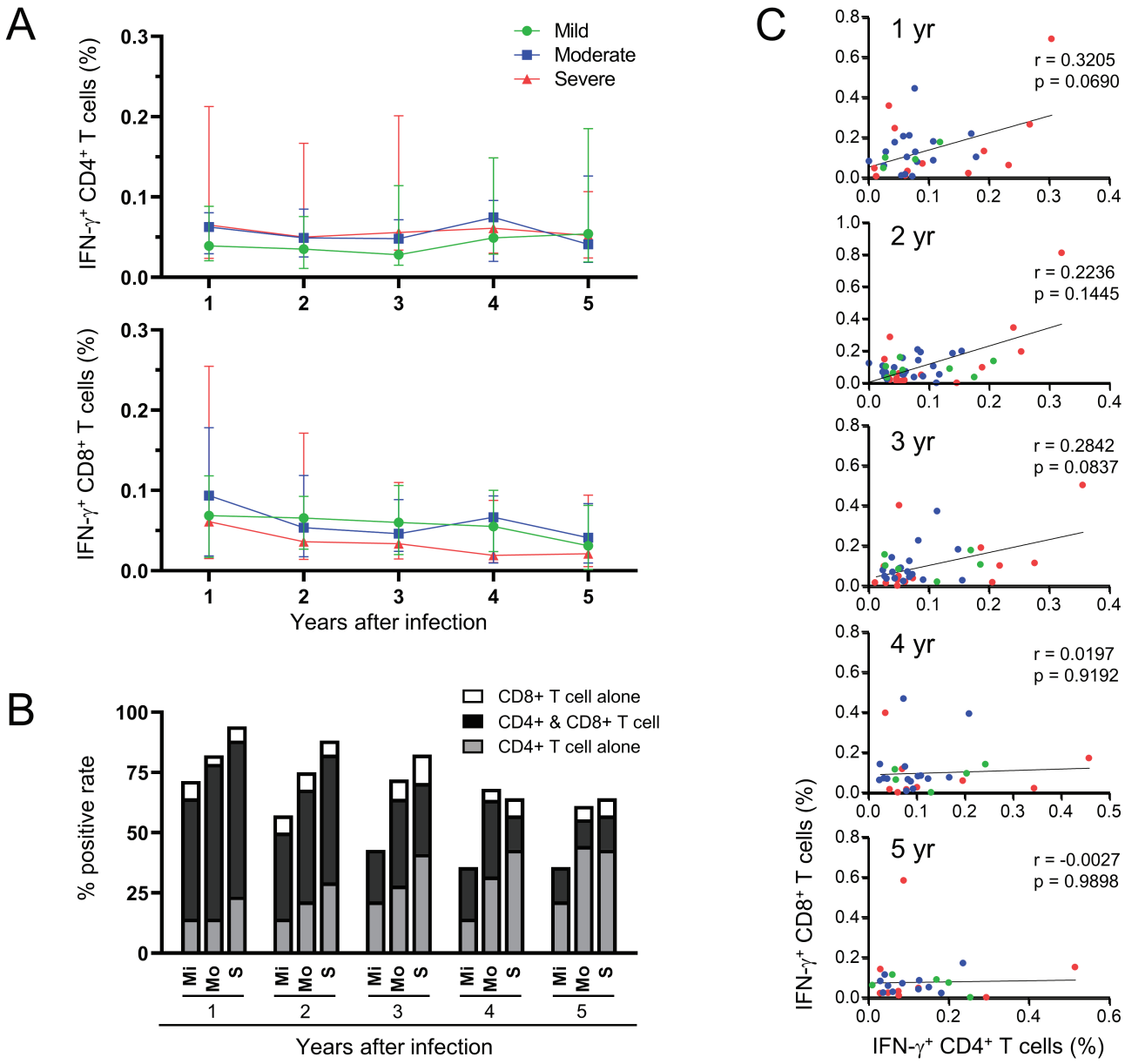

Figure 2. The relative contribution of CD4+ and CD8+ T-cell subset to memory T-cell responses. After stimulation of peripheral blood mononuclear cells with Middle East respiratory syndrome-coronavirus (MERS-CoV) peptide pools, IFN-γ–producing T cells were analyzed using intracellular cytokine staining. *A,* The frequency (median and interquartile range) of IFN-γ–producing CD4+ and CD8+ T cells in response to the 3 MERS-CoV peptide pools is shown. B, The positive rate of memory T-cell responses contributed by CD4+ and CD8+ T-cell subsets alone or together. *C,* Correlation between the frequency of antigen-specific CD4+ and CD8+ T cells in individual participants each year. The data were analyzed using the Spearman correlation. The number of participants used for this analysis per year was the same as that described in Figure 1. Abbreviation: IFN-γ, interferon gamma.

Functional Characteristics of MERS-CoV–Specific Memory T Cells

T cells that secrete multiple cytokines are considered superior in the control of viral infection [5]. Therefore, we addressed the proportion of single- or multiple-cytokine–secreting cells in MERS-CoV–reactive T cells. The functional subsets of virus-reactive CD4+ T cells were more or less evenly distributed among single-, double-, and triple-cytokine secretors, while single-cytokine–secreting cells were slightly dominant in virus-reactive CD8+ T cells (47%–72%); this distribution pattern tended to remain at all time points examined (Figures 4A and B). Approximately 2 of 3 virus-reactive CD8+ T cells produced IFN-γ alone or together with tumor necrosis factor alpha. Overall, there was no difference in the proportion of functional subsets in both antiviral CD4+ and CD8+ T cells among the groups with different clinical severities (Figures 4A and B).

Correlations Between Memory T-Cell Responses and Other Immune/Clinical Modalities

We addressed whether the magnitude of viral load detected at the acute stage of MERS-CoV infection could influence the

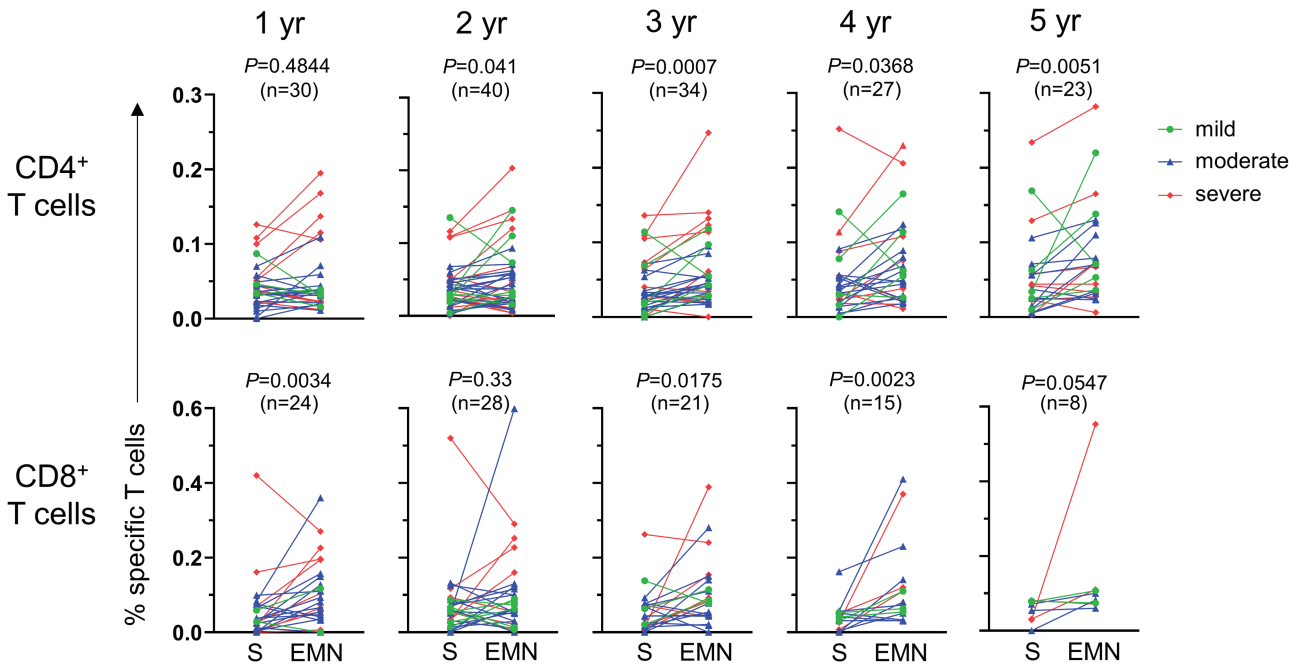

Figure 3. Reactivity of T cells to different viral proteins in individual participants. The lines indicate the paired frequencies of interferon gamma–producing T cells in response to the S1 or S2 pool of peptides and the E/M/N peptide pool in individual participants. This analysis included only participants with positive T-cell responses, and the number of participants is shown in each graph. The 2-tailed Wilcoxon signed-rank test was used to compare the paired samples.

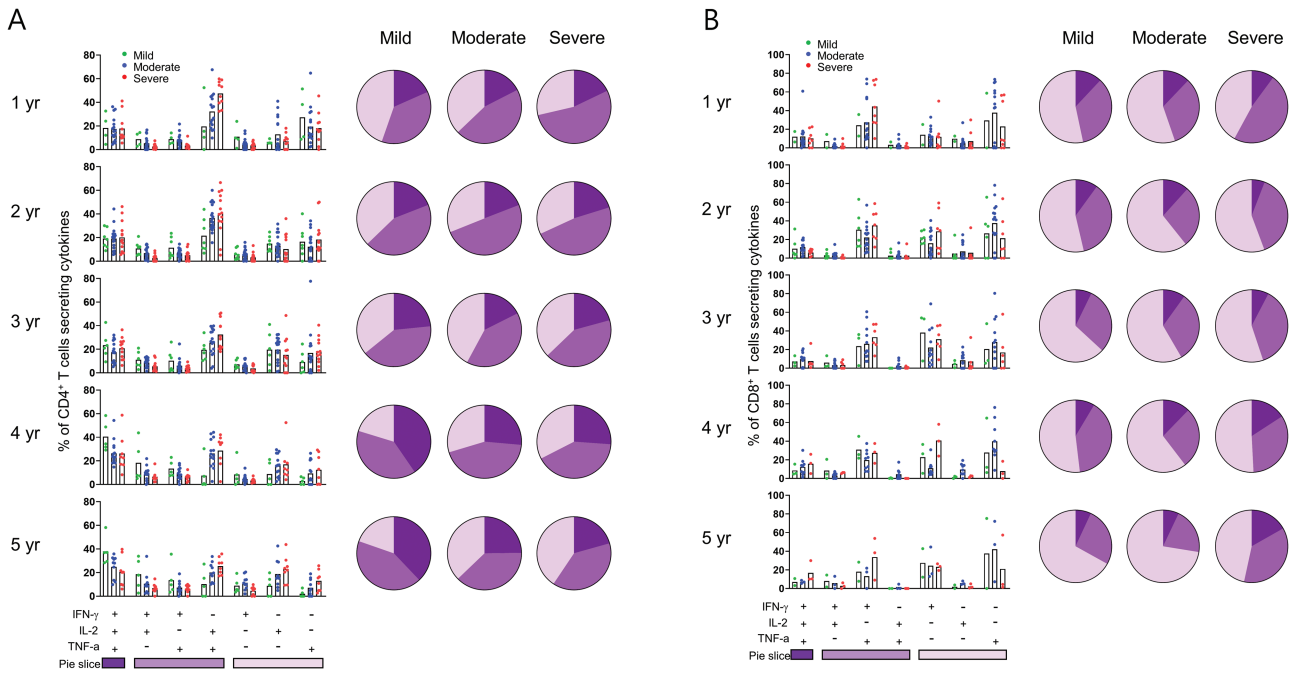

Figure 4. Polyfunctionality of Middle East respiratory syndrome-coronavirus (MERS-CoV)–specific T cells. Cytokine-production profiles of the MERS-CoV–specific CD4+ T cells and CD8+ T cells in each clinical severity group are shown in A and B, respectively. All possible combinations of 3 cytokines (IFN-γ, IL-2, and TNF-α) are shown on the *x*-axis. The bars indicate the average percentage of the total response contributed by T cells with a combination of responses on the *x*-axis. The responses are grouped according to the number of functions and data summarized using pie charts. Each slice of the pie represents the fraction of the total response that consists of T cells positive for a given number of functions. The mean data from participants with positive T-cell responses in each group are shown. Abbreviations: IFN-γ, interferon gamma; IL-2, interleukin 2; TNF-α, tumor necrosis factor alpha.

development of memory T-cell responses. Our analysis demonstrated that there was no association between the maximum viral titer and the frequency of virus-reactive T cells observed using either ELISPOT assay or ICS at any time point after infection (Figure 5A Supplementary Figures 5A and 6A). In contrast, when memory T-cell frequencies were plotted with the level of serum antibodies, anti-S1 immunoglobulin G (IgG), or nAbs, we found a highly significant relationship between them in the first 3 years after infection. However, the degree of this correlation gradually decreased over time, and anti-S1 IgG and nAb titers did not correlate with memory T-cell frequencies after the fourth and fifth years post-infection, respectively (Figure 5B). Interestingly, although virus-reactive CD4+ T-cell frequency correlated significantly with anti-S1 IgG responses longer than CD8+ T cells (3 years vs 2 years), virus-reactive CD8+ T-cell frequency correlated with nAb titers across all 5 years but CD4+ T cells only for 3 years after infection (Supplementary Figures 5B and 6B).

DISCUSSION

The fate of immune responses following an infection is critical for preparing effective control measures against a newly occurring viral infection. Our study demonstrates that functional memory T-cell responses following MERS infection lasted more than 5 years in 64% of the infected patients, and the maintenance of memory T cells was longer in patients with severe infection than in those with mild infection. Both CD4+ and CD8+ T cells, either alone or together, participated in this memory T-cell response to MERS-CoV, with the tendency that the initial magnitude of the response was slightly higher, but the longevity was shorter in CD8+ T cells than in CD4+ T cells. In addition, more T cells responded to the E/M/N viral proteins than to the S protein in both CD4+ and CD8+ T-cell subsets throughout the 5 years post-infection.

Human T-cell responses to acute viral infection were well explored in a longitudinal analysis of T cells responding to live yellow fever virus and smallpox vaccination [7, 8]. According to these studies, antiviral T cells greatly expanded during the second week of infection and then contracted abruptly over the next 2 weeks, followed by a gradual reduction thereafter, with a half-life of approximately 8–15 years. The kinetics of virus-specific T cells elicited by MERS-CoV infection seem to be similar to those observed in these model infections. Our previous study demonstrated that a high frequency of virus-specific CD4+ and CD8+

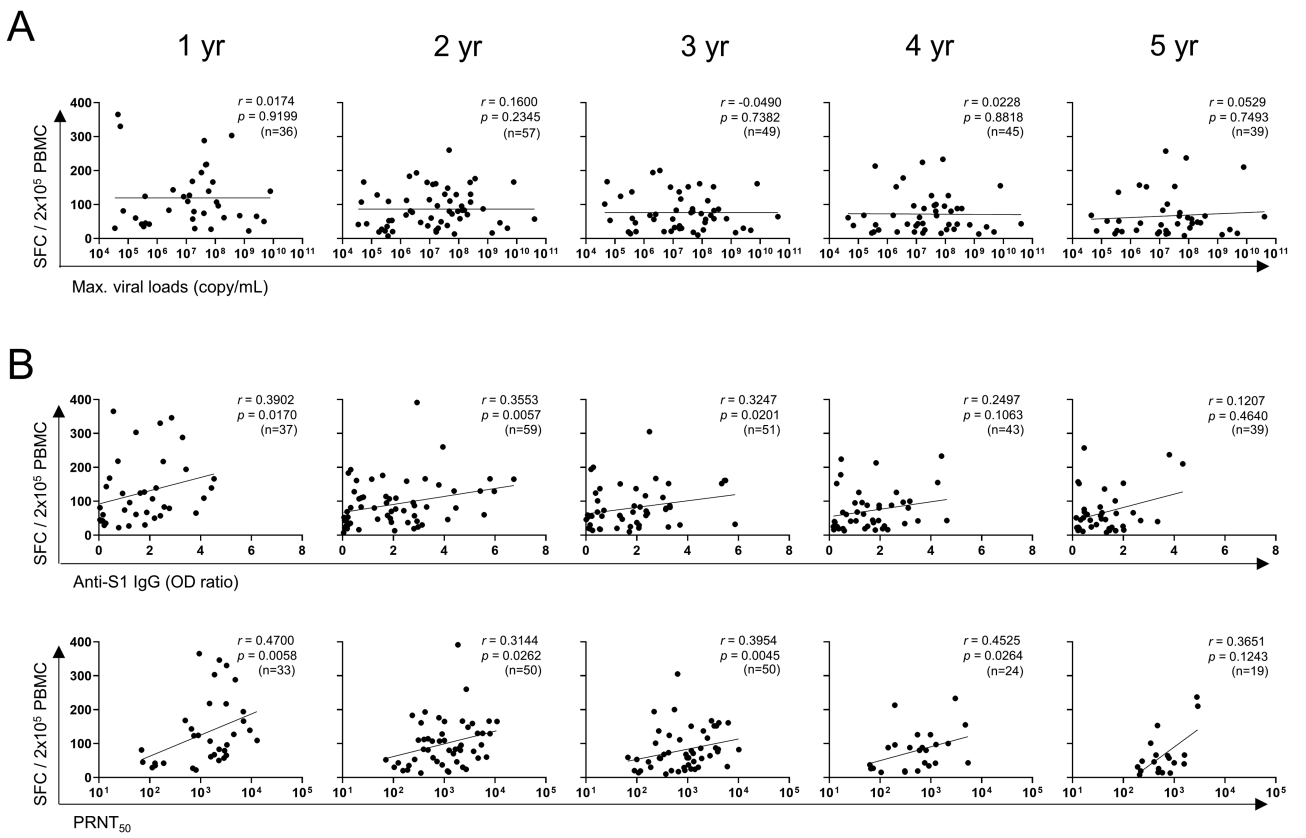

Figure 5. Correlation of the magnitude of memory T-cell response with maximum viral loads during the acute stage of infection or the level of specific antibodies. Correlations of the magnitude of memory T-cell response measured using enzyme-linked immunospot assay with maximum viral loads during the acute stage of infection *(A)* and the level of specific antibodies (anti-S1 IgG titer and $PRNT_{50}$) in serum samples collected each year *(B)* (data already published in ref [6]) were accessed using linear regression and the Spearman rank test. The numbers of participants used for this analysis are shown in each graph. Abbreviations: IgG, immunoglobulin G; OD, optical density; PBMC, peripheral blood mononuclear cell; $PRNT_{50}$, 50% plaque reduction neutralization test; SFC, spot-forming cell.

T cells (0.3% and 1.2% of mean percentage, respectively) were detected in most patients with MERS at the convalescent phase of acute infection (2–5 weeks after symptom onset) [4]. These cells greatly decreased at 9–12 months after infection (0.067% and 0.11% of the mean, respectively), as observed in the current study. Thereafter, a slow and gradual decline in virus-specific T cells was detected over the following 4 years. Interestingly, T-cell responses elicited by MERS vaccination in human clinical trials were also shown to follow kinetics similar to those induced by natural infection, at least during the first year after vaccination [9]. Based on the finding that memory T cells could be detected up to 17 years following infection with severe acute respiratory syndrome coronavirus (SARS-CoV) [10], which is a similar pathogenic coronavirus, memory T cells for MERS-CoV also seem to be long-lasting. Unexpectedly, a few participants maintained a relatively large number of memory T cells in their peripheral circulation throughout the entire observation period in our study. Although antigen persistence and memory inflation are known to cause the maintenance of high levels of memory T cells for a prolonged period [11], these mechanisms are not likely to work on our extraordinary observation because MERS-CoV does not cause persistent or chronic infection in humans. In contrast, cross-reaction eliciting from the infection with closely related human coronaviruses, such as OC43 and HKU1 [12], or a stochastic clonal expansion of memory T cells [13] could lead to the maintenance of high levels of or even rising T-cell memory responses. Further studies are needed to delineate the exact underlying mechanism of this observation.

Antibody response against MERS-CoV persisted in 86% of patients for at least 34 months [14] and in 36% in the fifth year after infection [6]. The magnitude and persistence of humoral immune responses in patients with MERS have been shown to correlate with the severity of viral infection [4, 6, 15]. Our study revealed that memory CD4+ T-cell responses also tended to have a positive relationship with clinical severity. However, antiviral CD8+ T cells showed the opposite behavior, a similar or lower frequency and shorter duration in the severe group than in the mild group. A previous study [16] indicated that antiviral T cells, particularly CD8+ T cells, could be detected even in mild MERS patients with undetectable antibody responses. A similar finding was reported in patients with COVID-19 [17]. Similarly, some patients (approximately 22%) showed positive CD8+ T-cell responses in the absence of antibody response in the first year after infection in our study. Nevertheless, it is of note that T-cell responses are longer-lasting than antibodies in MERS survivors, which is contributed mainly by the CD4+ T-cell subset rather than the CD8+ T-cell subset.

The maximum virus titer could be used as an indirect indicator of the amount of antigen load in viral infections and was associated with the severity of disease in our cohort (data not shown). The level of antibody responses 1 year after infection is positively correlated with the maximum viral loads during the acute stage of infection [18]. However, the level of memory T-cell responses did not correlate with the maximum viral loads at any time point, including 1 year after infection in our study, despite its positive correlation with antibody responses. Although we cannot rule out the possibility that the level of T-cell responses at the acute stage of infection could correlate with peak viral loads, our data revealed that exposure to high titers of the virus at the acute stage of infection does not necessarily guarantee the generation of high levels of long-term memory T lymphocytes.

Antiviral treatments with chemotherapeutic agents or immune modulators administered during the acute stage of infection could affect the persistence and magnitude of memory T-cell responses by either decreasing the viral burden or by preventing T-cell apoptosis, respectively [19, 20]. Most of our patients with severe/moderate illness received interferon and ribavirin, whereas less than half of the patients with mild illness did. This differential application of antiviral treatment could produce a differential development of memory T-cell responses in patients with different clinical severities. However, our limited analysis did not support this possibility because there was no significant difference in the magnitude of T-cell responses between treated and nontreated patients in either the mild or moderate illness group. Furthermore, the seemingly different persistence of memory T-cell responses observed in the comparative analysis among different severity level groups including all patients was still detected in the same analysis targeting only patients who received the same treatments. The 3 severely ill patients who received convalescent serum therapy did not show any specific difference in memory T-cell responses compared with the remaining patients with severe illness.

Memory T-cell responses following MERS-CoV infection observed in our study are similar to those following SARS-CoV infection in several aspects. First, the frequency of virus-specific T cells, especially CD4+ T cells, was higher in the severe group than in the mild/moderate group [21, 22]. In addition, the functionality of viral antigen-specific CD4+ and CD8+ T cells was comparable in recovered patients with various disease severities [21]. Furthermore, memory T cells could be detected in approximately 60% of SARS survivors at 6 years post-infection [22], implying similar longevity of memory T cells in patients with MERS and SARS. However, while SARS-CoV–specific CD4+ T cells were shown to respond predominantly to the S protein [21], MERS-CoV–reactive CD4+ T cells responded slightly higher to the E/M/N proteins than to the S protein. According to recent studies [23, 24], the overall immune responses to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, which is causing the current pandemic, do not appear to be much different from those revealed in SARS or MERS virus infection. If so, our current findings suggest that long-lasting memory T-cell responses could be attainable from SARS-CoV-2 infection or vaccination.

The limitations of this study include an uneven sample size across all time points, including the small number of first-year samples, especially in the mild group, due to the poor quality of frozen cells and increasing dropout on the progression of follow-up. Together with the heterogeneity of the measures depending on individual participants, the variation in sample size makes it difficult to reach a clear conclusion. In addition, further research is required to define the exact nature of extraordinarily strong T-cell responses to MERS-CoV peptide pools observed in some MERS survivors, as this could have a great impact on the interpretation of memory T-cell responses. Nonetheless, this study provides valuable information on the longevity and characteristics of memory T-cell responses attained from MERS-CoV infection in humans.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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