Clinical Infectious Diseases

MAJOR ARTICLE

MERS-CoV Infection Elicits Long-lasting Specific Antibody, T and B Cell Immune Responses in Recovered Individuals

Rowa Y. Alhabbab,[1,2,a] Abdullah Algaissi,[3,a] Ahmed Bakr Mahmoud,[4,a] Almohanad A. Alkayyal,[5,a] Sawsan Al-Amri,[1] Mohamed A. Alfaleh,[1,6] Mohammad Basabrain,[1] Roua Abdullah Alsubki,[7] Ibrahim S. Almarshad,[8] Abdulelah M. Alhudaithi,[8] Omar A. Al Gafari,[8] Yasser A. Alshamlan,[8] Hassan M. Aldossari,[8] Mohammed M. Alsafi,[8] Abdullah Bukhari,[9] Wael Bajhmom,[8] Ziad A. Memish,[10,11,12] Waleed S. Al Salem,[13] and Anwar M. Hashem[14]

[1]Vaccines and Immunotherapy Unit, King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia; [2]Department of Medical Laboratory Technology, Faculty of Applied Medical Sciences, King Abdulaziz University, Jeddah, Saudi Arabia; [3]Department of Medical Laboratory Sciences, Faculty of Applied Medical Sciences, Jazan University, Jazan, Saudi Arabia; [4]Department of Medical Laboratory Technology, College of Applied Medical Sciences, Taibah University, Almadinah Almunwarah, Saudi Arabia; [5]Department of Medical Laboratory Technology, College of Applied Medical Sciences, University of Tabuk, Tabuk, Saudi Arabia; [6]Department of Pharmaceutics, Faculty of Pharmacy, King Abdulaziz University, Jeddah, Saudi Arabia; [7]Department of Clinical Laboratory Science, College of Applied Medical Sciences, King Saud University, Saudi Arabia; [8]Ministry of Health, Riyadh, Saudi Arabia; [9]Department of Medicine, Faculty of Medicine, Imam Mohammed Ibn Saud Islamic University, Riyadh, Saudi Arabia; [10]King Saud Medical City, Research & Innovation Center, Ministry of Health, Riyadh, Saudi Arabia; [11]Al-Faisal University, Riyadh, Saudi Arabia; [12]Hubert Department of Global Health, Rollins School of Public Health, Emory University, Atlanta, Georgia, USA; [13]Department of Agriculture, Ministry of Environment, Water and Agriculture, Riyadh, Saudi Arabia; and [14]Department of Medical Microbiology and Parasitology, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia

Background. The Middle East respiratory syndrome coronavirus (MERS-CoV) is a highly pathogenic zoonotic betacoronavirus and a global public health concern. Better undersetting of the immune responses to MERS-CoV is needed to characterize the correlates of protection and durability of the immunity and to aid in developing preventative and therapeutic interventions. Although MERS-CoV–specific circulating antibodies could persist for several years post-recovery, their waning raises concerns about their durability and role in protection. Nonetheless, memory B and T cells could provide long-lasting protective immunity despite the serum antibodies levels.

Methods. Serological and flow cytometric analysis of MERS-CoV–specific immune responses were performed on samples collected from a cohort of recovered individuals who required intensive care unit (ICU) admission as well as hospital or home isolation several years after infection to characterize the longevity and quality of humoral and cellular immune responses.

Results. Our data showed that MERS-CoV infection could elicit robust long-lasting virus-specific binding and neutralizing antibodies as well as T- and B-cell responses up to 6.9 years postinfection regardless of disease severity or need for ICU admission. Apart from the persistent high antibody titers, this response was characterized by B-cell subsets with antibody-independent functions as demonstrated by their ability to produce tumor necrosis factor α (TNF-α), interleukin (IL)-6, and interferon γ (IFN-γ) cytokines in response to antigen stimulation. Furthermore, virus-specific activation of memory $CD8^+$ and $CD4^+$ T cell subsets from MERS-recovered patients resulted in secretion of high levels of TNF-α, IL-17, and IFN-γ.

Conclusions. MERS-CoV infection could elicit robust long-lasting virus-specific humoral and cellular responses.

Keywords. coronaviruses; MERS-CoV; T cells; antibodies; longevity; immunity.

Three novel zoonotic coronaviruses (CoVs) have emerged in the past 2 decades leading to a significant impact on global public health. The ongoing global coronavirus disease 2019 (COVID-19) pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the last example of such threat. In 2002, severe acute respiratory syndrome coronavirus 1 (SARS-CoV-1) emerged in China and rapidly spread globally, leading to >8000 infections and ~800 deaths [1, 2]. Ten years later, the Middle East respiratory syndrome coronavirus (MERS-CoV) was discovered as a new betacoronavirus in Saudi Arabia in 2012 and has since resulted in ~2580 confirmed cases and ~890 deaths (~34.5% mortality rate) in 27 countries [3, 4]. Unlike SARS-CoV-1, which disappeared since 2004, MERS-CoV continues to circulate causing a continuous global public health concern. Current epidemiological evidence indicates that dromedaries are MERS-CoV reservoir host and the main source of primary human zoonotic infections [5, 6].

Among the key outstanding questions in CoV infection yet to be addressed is whether these infections could induce long-lasting protective immunity. Thus, better understanding of the durability of the immune response against CoV infection is important not only for vaccines and therapeutics development but also for our understanding of the immune correlates of protection, disease

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[a]R. Y. A., A. A., A. B. M, A. A. A. contributed equally to this work.

Correspondence: A. M. Hashem, King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia (amhashem@kau.edu.sa).

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Table 1. Demographics of MERS Recovered Individuals

Patient ID	City	Gender	Age	Isolation, ICU	Year of Infection	Years Since Infection
R1	Buridah	F	29	Home isolation	2016	4.6
R2	Buridah	M	49	ICU	2015	5.6
R3[a]	Buridah	M	61	ICU	2016	4.6
R4[a]	Um Aljowa	M	39	Hospital isolation	2018	2.7
R5	Buridah	M	43	Home isolation	2019	1.2
R6	Buridah	M	30	Hospital isolation	2016	4.7
R7[a]	Buridah	M	43	Hospital isolation	2016	4.7
R8[a]	Buridah	M	67	Hospital isolation	2016	4.8
R9[a]	Buridah	F	97	Hospital isolation	2017	3.8
R10[a]	Buridah	M	43	Hospital isolation	2016	4.8
R11[a]	Buridah	M	39	Hospital isolation	2015	5.8
R12[a]	Buridah	M	43	Hospital isolation	2016	4.8
R13[a]	Jeddah	M	55	ICU	2014	6.6
R14[a]	Jeddah	M	63	ICU	2014	6.9
R15	Jeddah	M	37	Home isolation	2014	6.7
R16	Jeddah	M	26	ICU	2014	6.6
R17	Jeddah	M	65	Home isolation	2014	6.7
R18	Jeddah	M	36	ICU	2019	2.0
R19	Jeddah	M	41	Home isolation	2019	2.0
R20	Wadi Aldawser	M	32	Hospital isolation	2019	1.9
R21	Wadi Aldawser	F	42	Hospital isolation	2019	2.0

Abbreviation: ICU, intensive care unit.

[a]Indicates individuals recruited for peripheral blood mononuclear cell (PBMC) collection that was used for B- and T-cell flow cytometry analysis.

transmission and severity. Although such questions are highly relevant to the ongoing COVID-19 pandemic, it's too early to study the longevity of immunity to SARS-CoV-2 as it has just recently emerged. Nonetheless, several studies have investigated memory responses against SARS-CoV-1 as it was the first highly pathogenic CoV to be associated with human disease. These studies have shown persistence of T-cell and antibody responses in patients recovered from SARS-CoV-1 infection up to 17 years [7–10], although other studies suggested potential waning of antibodies and B cells overtime to low or undetectable levels [11, 12]. Although limited number of studies have evaluated the longevity of immunity in MERS survivors, most of these studies have mainly focused on evaluating antibody responses which were found to last for up to 6 years post-recovery in most survivors [13]. Interestingly, such response was associated with disease severity and could wane over time especially in mild and asymptomatic cases [13–17]. On the other hand, T cells were only investigated during acute phase or after few months post-infection and found to be strong in severe and moderate cases [7, 18, 19]. In this study, we sought to determine the longevity of humoral and cellular immunity against MERS-CoV in recovered individuals up to 6.9 years after the initial infection.

METHODS

Clinical Samples

Heat inactivated serum samples were collected from MERS recovered individuals (n = 21) at different years post-recovery. Archived serum samples from healthy controls (n = 10) were used as unexposed negative controls. PBMCs were obtained from 10 MERS recovered individuals and 4 healthy controls. Written or verbal informed consents were obtained from all participants and the work was approved by Directorate of Health Affairs, Saudi Ministry of Health (Project number 1496).

Detection of MERS-CoV Binding IgG by ELISA

Detection of anti-nucleocapsid (N), -spikee subunit 1 (S1), and -receptorr. binding domain (RBD) IgG antibodies against MERS-CoV was performed as previously described [20, 21] using recombinant MERS-CoV N, spike S1 subunit and RBD proteins. Detailed method is available in Supplementary Materials and Methods.

Pseudovirus Neutralizing Antibody Assay

Generation of pseudovirus expressing MERS-CoV S based on the recombinant vesicular stomatitis virus (VSV) (rVSV-ΔG/MERS-S*-luciferase pseudovirus) and neutralization assay were performed as previously described [22]. Detailed method is available in Supplementary Materials and Methods.

Antigen-specific Memory B-Cell Detection

MERS-CoV S1 specific B cells in PBMCs were detected using biotinylated S1 protein multimerized with phycoerythrin labeled streptavidin (SA-PE). SA-PE without biotinylated MERS-CoV S1 was used to gate out MERS-CoV S1 nonspecific

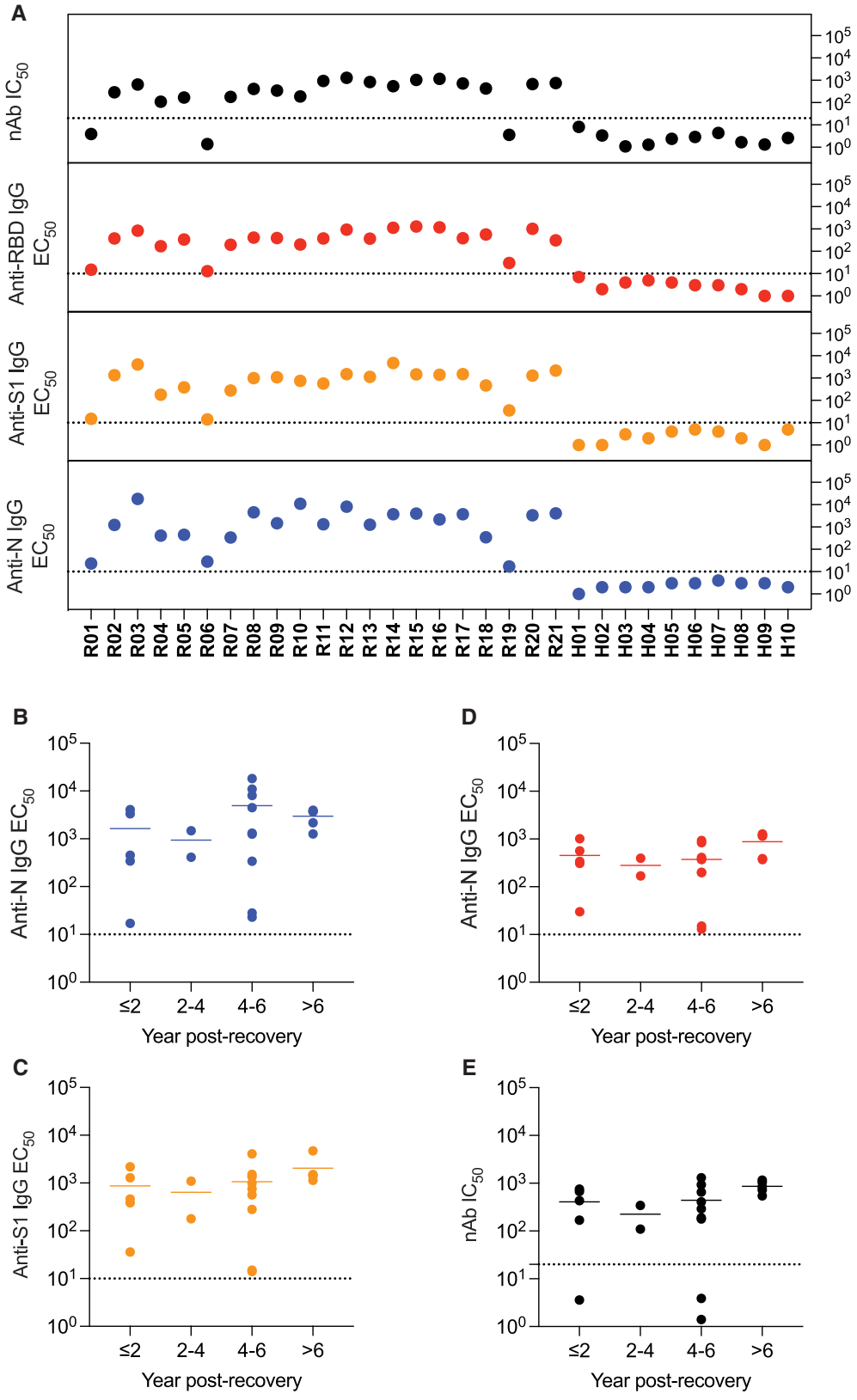

Figure 1. Antibody response in MERS survivors. *A*, Serum samples from MERS recovered (R) individuals (n = 21) and healthy donors (H) (n = 10) were tested by ELISA for anti-N, -S1 and -RBD IgG and titers were determined as EC_{50}. Neutralizing antibodies (nAbs) in serum samples were determined using pseudovirus assay and titers were determined as IC_{50}. *B–E*, Binding antibody titers and nAbs are reported against time of sample collection post-recovery. Mean is shown in *B–E* panels for each group. Abbreviations: ELISA, enzyme-linked immunosorbent assay; IC_{50}, half maximal inhibitory concentration; IgG, immunoglobulin G; MERS, Middle East respiratory syndrome; N, nucleocapsid; RBD, receptor binding domain; S1, spike subunit 1.

binding cells. Cells were stained with MERS-S1-PE, BV510-conjugated anti-CD19, PE-Cy7-conjugated anti-CD27, APC-conjugated anti-CD38, PerCP-Cy5.5-conjugated anti-CD138, PB-conjugated anti-IgD, and Alexa Fluor 700-conjugated anti-IgM antibodies. Cells were washed, fixed, and acquired using BD FACSAria™ III flow cytometer. Detailed method is available in Supplementary Materials and Methods.

Antigen-specific Memory B- and T-Cell Response Detection

PBMCs were restimulated in the presence of brefeldin A with either recombinant S1 protein (1 μg/mL) or 15-mer overlapping peptides pool covering the entire MERS-CoV S protein (5 μg/mL) to activate B or T cells, respectively. Incubation with phorbol myristate acetate/ionomycin or Roswell Park Memorial Institute (RPMI) 1640 medium served as positive and unstimulated controls, respectively. Cells were then stained with LIVE/DEAD stain and incubated with BV510-conjugated anti-CD19, PE-Cy7-conjugated anti-CD27, APC-conjugated anti-CD38, PE-CF594-conjugated anti-CD24, and PerCP-Cy5.5-conjugated anti-CD138 surface antibodies to stain B cells. T cells were stained with APC-Cy7-conjugated anti-CD3, BV510-conjugated anti-CD4, PE-Cy7-conjugated anti-CD8, PerCP-Cy5.5-conjugated anti-CD45RA, and APC-conjugated anti-CCR7 surface antibodies. Cells were then washed and fixed and permeabilized and intracellularly stained with PE-conjugated anti-IL-6, PE-conjugated anti-IL17α, FITC-conjugated anti-IFN-ɣ, and BV421-conjugated anti-TNF-α antibodies. Cells were washed, fixed, and acquired using BD FACSAria™ III flow cytometer. Detailed method is available in Supplementary Materials and Methods.

Statistical Analysis

Analyses and comparisons between groups using *t* test were performed using GraphPad Prism V9 software (GraphPad Co., San Diego, California, USA). Enzyme-linked immunosorbent assay (ELISA) and neutralization titers were reported as EC_{50} and IC_{50} values using 4-parameter logistic (4PL) curve in GraphPad Prism V9 software. All flow cytometry data were analyzed using FlowJo v10 software (Tree Star Inc., Ashland, Oregon, USA). All antibodies used were from BioLegend, UK.

RESULTS

Demographics of Study Participants

For this study, we recruited 21 individuals (18 males and 3 females) who were diagnosed based on positive reverse transcription polymerase chain reaction (RT-PCR) results and recovered from MERS infection. Participants represented a range of MERS cases who required intensive care unit (ICU) admission (n = 6), as well as hospital (n = 10) or home (n = 5) isolation only (Table 1). Subjects were recruited from multiple cities in Saudi Arabia (Table 1). The mean age for the participants was 46.7 ± 16.6 (range 26–97 years). The average time between sample collection and recovery was 4.5 ± 1.9 years (range 1.2–6.9 years).

MERS-CoV Circulating Antibodies

Assessment of durability of circulating MERS-CoV specific immunoglobulin G (IgG) antibody titers was performed using in-house enzyme-linked immunosorbent assays (ELISA) that have been previously validated and described by our group [20, 21]. As N protein is the most abundant protein in the virus, N–binding IgG titers were determined in all subjects. Also, MERS-CoV recombinant S1 subunit and RBD proteins, which contain the main epitopes targeted by most neutralizing antibodies (nAbs) against MERS-CoV, were used to measure antigen-specific binding IgG endpoint titers. MERS-CoV N IgG half-maximal binding (EC_{50}) titers were relatively stable, but they were heterogeneous among subjects (range 17–18 833; median 1472). All participants except for 3 showed high levels of N-binding IgG titers (Figure 1*A*). These 3 individuals were from the group that required home isolation only and never admitted to the hospital. Similarly, anti-S1 IgG EC_{50} titers were also stable and heterogeneous to a large extent (range 14–4729; median 1097). Although anti-RBD IgG EC_{50} titers were lower than that of S1-binding IgG (range 13–1281; median 374), they largely matched each other. The same 3 subjects with low anti-N IgG titers also showed very low titers of anti-S1 and RBD IgG. To evaluate the function of these antibodies, we also measured the levels of MERS-CoV nAbs in the serum using a vesicular stomatitis virus (VSV)-based pseudovirus assay as we have previously described [22]. As shown in Figure 1*A*, only individuals with high binding IgG levels showed positivity for nAbs with median inhibitory concentration (IC_{50}) titer of 598 (range 100–1300). As expected, no binding or nAbs were detected in archived serum samples from healthy controls. To further assess durability of the antibody response especially that longitudinal samples were not collected for each participant, we analyzed antibody titers against sample collection time post-recovery. As shown in Figures 1*B*–*E*, circulating binding and nAbs were maintained at high titers even in individuals sampled more than 6 years post-recovery. Together, these data suggest that recovery from MERS could result in persistent and long-lasting circulating binding and nAbs for more than 6 years postinfection in most subjects.

MERS-CoV Memory B Cells

To gain an idea of the expected frequency of MERS-specific memory B cells in peripheral blood in MERS recovered individuals, biotinylated MERS-S1 proteins were bound to fluorescently labeled streptavidin resulting in fluorescent labeled-probes that were used to determine the frequencies of S1-specific B-cell subsets from representative samples. This included non-class switched B cells defined as $CD19^+$ IgD^+IgM^+

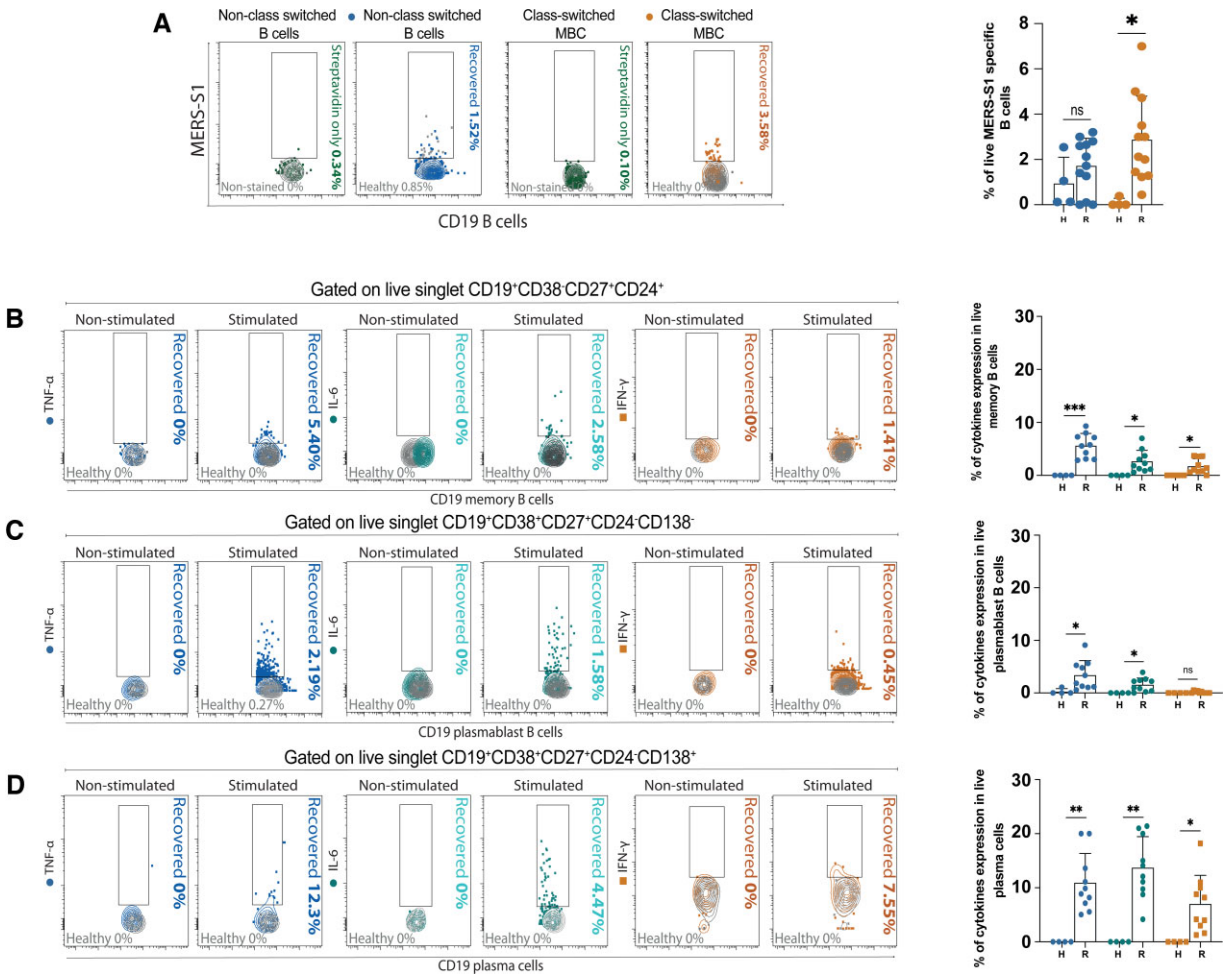

Figure 2. B-cell response in MERS survivors. PBMCs were isolated from MERS recovered individuals as well as healthy donors by lymphoprep density gradient centrifugation, and incubated with either (*A*) MERS-S1 conjugated to PE or (*B–D*) MERS-S1 protein and brefeldin A. *A*, Representative FACS plots of MERS-S1 specific non-class switched and memory B cells, and summary data showing the percentage of these specific B cell subsets in live cells in each cell subset from MERS recovered (R) individuals (n = 10) and healthy donors (H) (n = 4). Representative FACS plots of TNF-α, IL-6 and IFN-γ expression in stimulated and nonstimulated (*B*) memory B cells, (*C*) plasmablast B cells, and (*D*) plasma cells, and summary data showing TNF-α, IL-6, and IFN-γ expression in live cells in each B-cell subset from MERS recovered (R) individuals (n = 10) and healthy donors (H) (n = 4). Data are shown as mean ± SD for each group. Statistics were calculated by *t* test. $^{*}P < .05$, $^{**}P < .005$, $^{***}P = .0005$. Supplementary table shows the levels of these cell subsets from total lymphocytes population. Abbreviations: FACS, fluorescence-activated cell sorting; IFN-γ, interferon γ; IL-6, interleukin 6; MERS, Middle East respiratory syndrome; ns, not significant; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin, SD, standard deviation; TNF-α, tumor necrosis factor α.

B cells, class switched $CD19^{+}$ $CD27^{+}$ $IgD^{-}IgM^{-}CD38^{-}$ memory B cells (MBC) by flow cytometry (Supplementary Figure 1*A*). The frequency of MERS-S1-specific non-class switched B cells did not differ significantly between healthy controls and MERS recovered individuals (Figure 2*A*). On the other hand, the frequencies of MERS-S1-specific class switched MBC were significantly higher in MERS recovered participants in comparison to healthy individuals as expected (Figure 2*A*).

There is limited knowledge regarding antibody-independent memory B-cell responses in individuals following MERS infection. Thus, we next determined whether prolonged MERS-specific B-cell-mediated immune responses are persistent in MERS-recovered individuals. Therefore, peripheral blood mononuclear cells (PBMCs) from healthy controls and MERS-recovered individuals were restimulated ex vivo with recombinant MERS S1 protein for 16 hours. After restimulation, the levels of intracellular tumor necrosis factor α (TNF-α), interleukin-6 (IL-6), and interferon γ (IFN-γ) were assessed in the different subpopulations of B cells (Supplementary Figure 1*B*). Remarkably, flow cytometric analysis revealed that the levels of TNF-α, IL-6, and IFN-γ producing memory ($CD19^{+}CD38^{-}CD27^{+}CD24^{+}$) (Figure 2*B*) and plasma B cells ($CD19^{+}CD38^{+}CD27^{+}CD24^{-}CD138^{+}$) (Figure 2*D*) were significantly higher in MERS survivors compared to healthy donors after re-stimulation with MERS S1 protein. In addition, intracellular production of TNF-α and IL-6 in plasmablast B cells ($CD19^{+}CD38^{+}CD27^{+}CD24^{-}CD138^{-}$) from MERS-recovered individuals were significantly higher than healthy donors

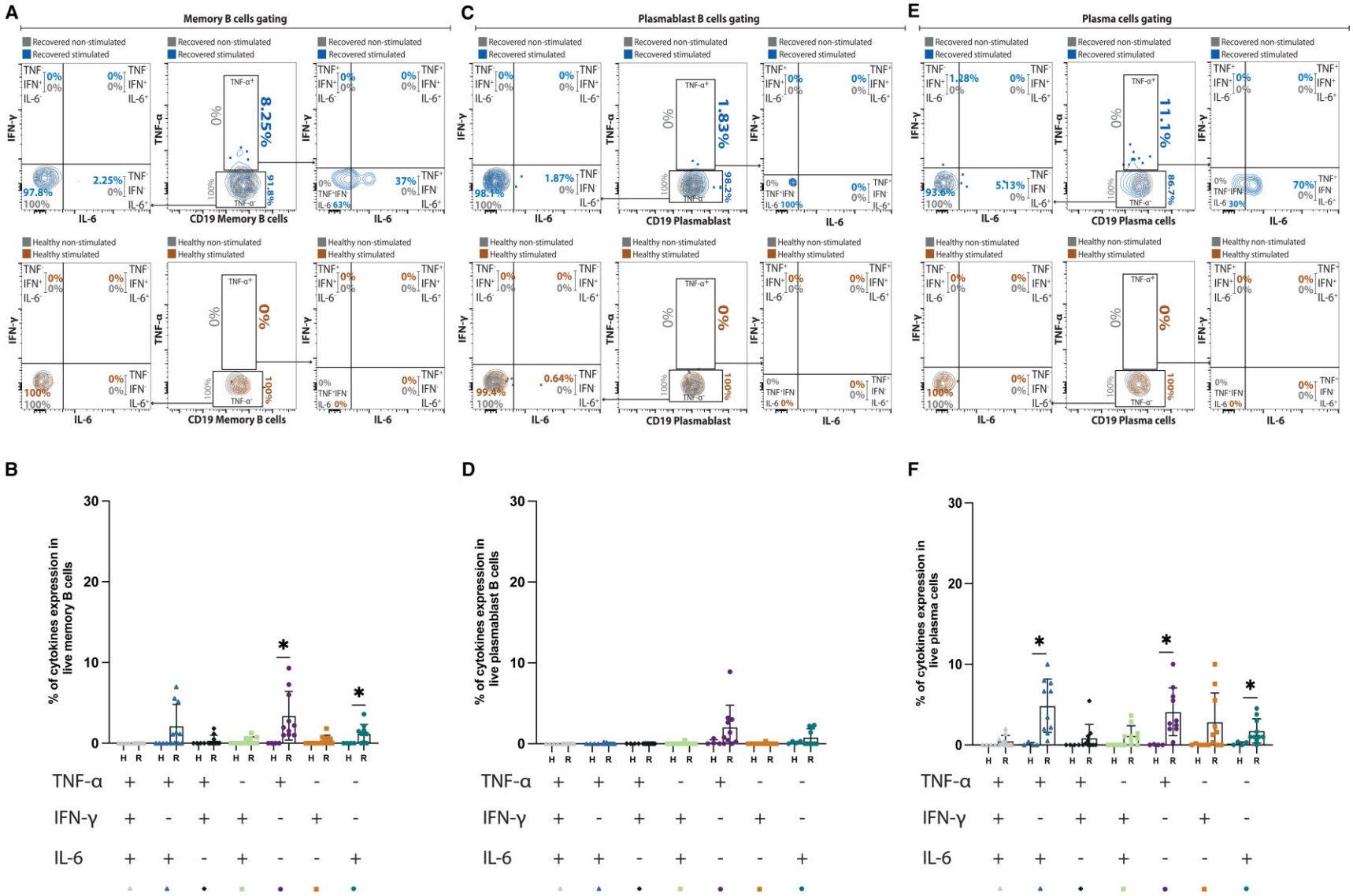

Figure 3. Single-, double- and triple-cytokine producing memory B cells. Representative FACS plots of single-, double- and triple-cytokine–producing cells in stimulated and nonstimulated (*A*) memory B cells ($CD19^+CD38^-CD27^+CD24^+$), (*C*) plasmablast B cells ($CD19^+CD38^+CD27^+CD24^-CD138^-$) and (*E*) plasma B cells ($CD19^+CD38^+CD27^+CD24^-CD138^+$) from MERS recovered (R) individuals and healthy donors (H). Bar graphs represent percentage of summary data showing single-, double-, and triple-cytokine expression in B-cell subsets (*B*) memory B cells, (*D*) plasmablast B cells, and (*F*) plasma B cells in MERS recovered (R) individuals and healthy donors (H). Data in histograms are shown as percentages of induced cytokines from restimulated cells after subtracting levels produced by unstimulated cells from each individual. Data are shown as mean ± SD for each group. Statistics were calculated by *t* test, $^*P < .05$. Abbreviations: FACS, fluorescence-activated cell sorting; IFN-γ, interferon γ; IL-6, interleukin 6; MERS, Middle East respiratory syndrome; SD, standard deviation; TNF-α, tumor necrosis factor α.

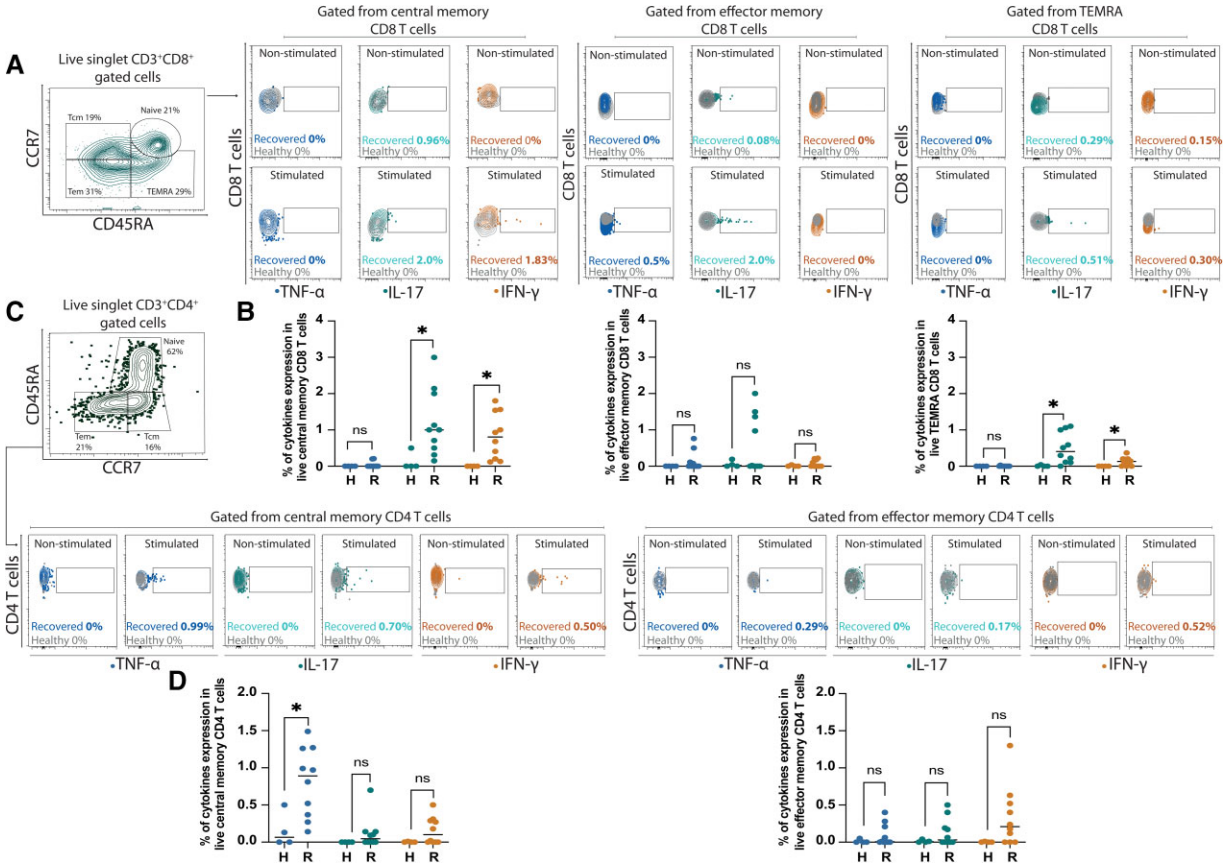

Figure 4. Memory T-cell response in MERS recovered individuals. PBMCs were isolated from MERS recovered individuals as well as healthy donors by lymphoprep density gradient centrifugation, and *ex vivo* re-stimulated with MERS-CoV S overlapping peptide pool. *A*, Representative FACS plots of TNF-α, IL-17, and IFN-γ expression from stimulated and non-stimulated $CD3^+CD8^+CCR7^+CD45RA^-$ central memory, $CD3^+CD8^+CCR7^-CD45RA^-$ effector memory and $CD3^+CD8^+CCD7^-CD45RA^+$ TEMRA T cells from MERS recovered individuals and healthy donors. *B*, Summary data showing the expression of TNF-α, IL-17, and IFN-γ among live $CD8^+$ memory T-cell subsets in MERS recovered (R) individuals (n = 10) and healthy donors (H) (n = 4). *C*, Representative FACS plots of TNF-α, IL-17 and IFN-γ expression from stimulated and non-stimulated $CD3^+CD4^+CCR7^+CD45RA^-$ central and $CD3^+CD4^+CCR7^-CD45RA^-$ effector memory T cells from MERS recovered individuals and healthy donors. *D*, Summary data showing the expression of TNF-α, IL-17, and IFN-γ in live $CD4^+$ memory T-cell subsets in MERS recovered (R) individuals (n = 10) and healthy donors (H) (n = 4). Mean is shown for each group. Statistics were calculated by *t* test, $^*P < .05$. Supplementary table shows the levels of these cell subsets from total lymphocytes population. Abbreviations: FACS, fluorescence-activated cell sorting; IFN-γ, interferon γ; IL-17, interleukin 17; MERS, Middle East respiratory syndrome; ns, not significant; SD, standard deviation; TNF-α, tumor necrosis factor α.

(Figure 2*C*). In contrast, IFN-γ was not detectable in $CD19^+$ plasmablast B cells (Figure 2*C*). These cytokines were mainly produced by S1-specific single-cytokine producing B cells, although some double-cytokine producing cells were detected in memory and plasma B cells (Figure 3). Overall, our results indicate that MERS infection induces robust and long-lived MERS-specific memory B cells, which could confer prolonged and efficient immune protection.

MERS-CoV Specific T-Cell Response

T cells in principle can contribute to a rapid clearance of CoVs infection and induce long-lasting cellular responses. To further delineate this response in MERS-recovered persons, we evaluated S-specific $CD8^+$ and $CD4^+$ T cells. Specifically, we restimulated PBMCs from MERS survivors and healthy individuals with a pool of overlapping peptides covering antigenic epitopes of MERS-CoV S protein and assessed antigen-specific secreted levels of TNF-α, IL-17, and IFN-γ from $CD8^+$ and $CD4^+$ T-cell subpopulations by flow cytometry. Specifically, we phenotypically characterized the different subsets of T cells including $CD8^+$ T central memory cells ($CD8^+$ Tcm; $CD3^+CD8^+CCR7^+CD45RA^-$), $CD8^+$ T effector memory cells ($CD8^+$ Tem; $CD3^+CD8^+CCR7^-CD45RA^-$), and $CD8^+$ T terminally differentiated effector cells ($CD8^+$ TEMRA; $CD3^+CD8^+CCR7^-CD45RA^+$), in addition to $CD4^+$ T central memory cells ($CD4^+$ Tcm; $CD3^+CD4^+CCR7^+CD45RA^-$) and $CD4^+$ T effector memory cells ($CD4^+$ Tem; $CD3^+CD4^+CCR7^-CD45RA^-$) (Supplementary Figure 2). Our results showed that both $CD8^+$ and $CD4^+$ T cells from MERS-recovered patients were able to produce TNF-α, IL-17,

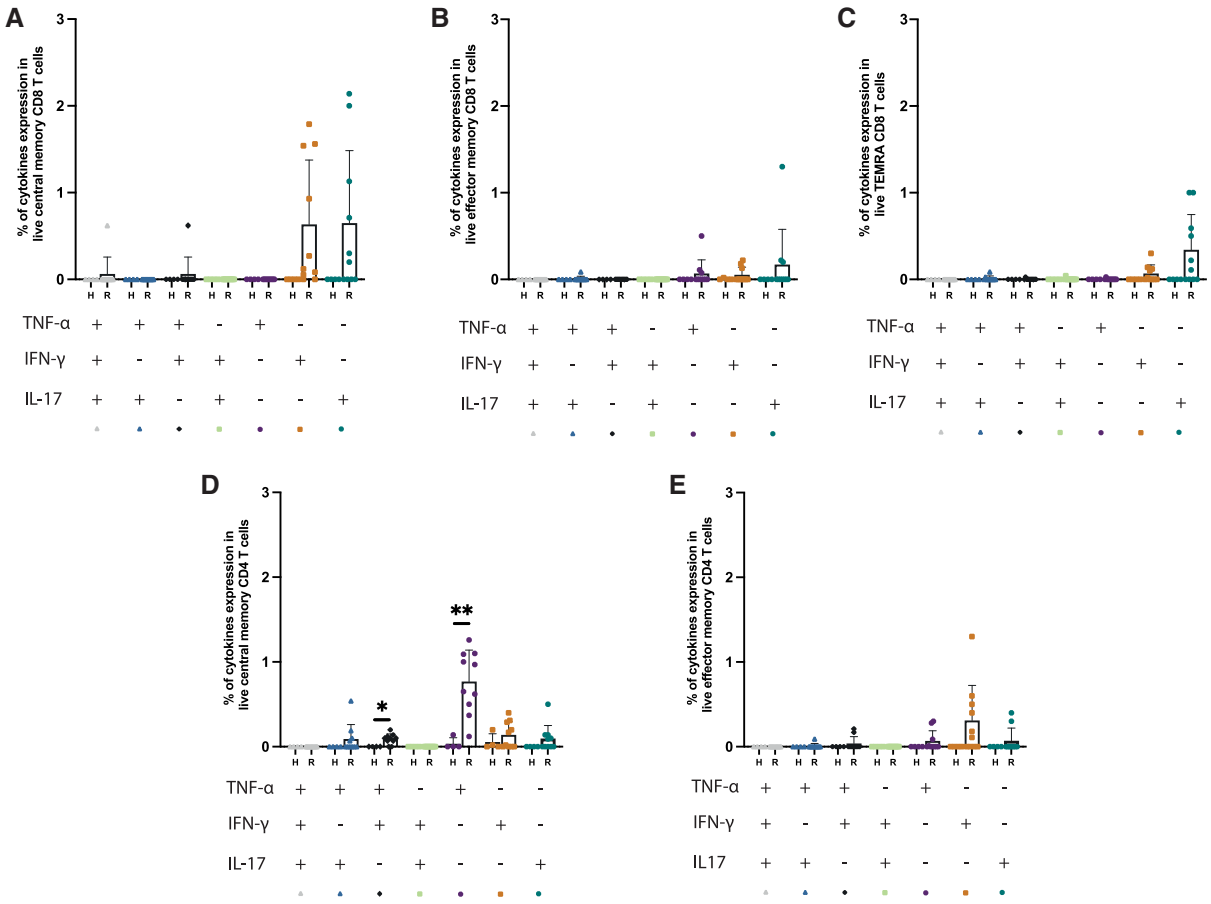

Figure 5. Single-, double-, and triple-cytokine producing memory T cells. Bar graphs represent percentage of single-, double- and triple-cytokine–producing (*A*) central memory $CD8^+$ T cells ($CD3^+CD8^+CCR7^+CD45RA^-$), (*B*) effector memory $CD8^+$ T cells ($CD3^+CD8^+CCR7^-CD45RA^-$), (*C*) terminally differentiated effector $CD8^+$ T cells ($CD3^+CD8^+CCR7^-CD45RA^+$), (*D*) central memory $CD4^+$ T cells ($CD3^+CD4^+CCR7^+CD45RA^-$), and (*E*) effector memory $CD4^+$ T cells ($CD3^+CD4^+CCR7^-CD45RA^-$). Data in histograms are shown as percentages of induced cytokines from re-stimulated cells after subtracting levels produced by unstimulated cells from each individual. Data are shown as mean ± SD for each group. Statistics were calculated by *t* test, $^*P < .05$. Representative FACS plots of single-, double-, and triple-cytokine–producing cells in stimulated and nonstimulated from MERS recovered (R) individuals and healthy donors are shown in Supplementary Figure 3. Abbreviations: FACS, fluorescence-activated cell sorting; IFN-γ, interferon γ; IL-17, interleukin 17; MERS, Middle East respiratory syndrome; SD, standard deviation; TNF-α, tumor necrosis factor α.

and IFN-γ in the presence of MERS-CoV S1 peptide pool as compared to healthy controls. The percentage of IL-17 and IFN-γ producing $CD8^+$ Tcm and TEMRA cells from MERS-recovered individuals were significantly higher than that from healthy individuals, whereas no statistical difference was observed in the levels TNF-α-producing $CD8^+$ T cells between those two groups (Figures 4*A* and 4*B*). Interestingly, no significant differences were observed in the levels of cytokines produced by $CD8^+$ Tem cells. Moreover, TNF-α producing $CD4^+$ Tcm cells were significantly higher in MERS survivors, and this was not the case for $CD4^+$ Tem cells (Figure 4*C* and 4*D*). Although few MERS-recovered individuals showed some high levels of IL-17 and IFN-γ producing $CD4^+$ Tem and Tcm cells, no statistical differences were observed (Figure 4*C* and 4*D*). Notably, most of the cytokines produced by both $CD8^+$ and $CD4^+$ T cells subpopulations were produced by single-cytokine producing cells and not polyfunctional T cells (Figure 5 and Supplementary Figure 3). Taken together, T-cell characterization results indicated that MERS-specific $CD8^+$ and $CD4^+$ memory T cells are persistent and could play a significant role in the cellular immune responses against infection in MERS survivors and that this response is heterogeneous with a broad spectrum of functions of each T-cell subset.

DISCUSSION

Among the consequential unknowns of the highly pathogenic human CoVs are the durability and quality of the immune responses. Although such data are critical not only for understanding the immune response and the likelihood of reinfections but also for the development of effective vaccines

and therapeutics, there are limited data on long-term immunity after SARS-CoV-1, MERS-CoV, and SARS-CoV-2 infections. Here we characterized the longevity of the humoral and cellular immune responses up to 6.9 years post MERS-CoV infection in recovered individuals from Saudi Arabia. Our data showed that MERS-CoV specific IgG and nAb responses could be sustained for up to 6.9 years after infection in most recovered individuals. We found a robust and long-lived MERS-CoV-specific memory B cells as well as $CD4^+$ and $CD8^+$ T cells responses in these individuals, which could confer prolonged and efficient immune protection. In general, these findings are consistent with previous studies conducted on smaller sample sizes and after shorter periods after initial MERS infection [7, 13–19].

The hallmark of the immune response durability is the formation of long-term memory immune cells including memory B and T lymphocytes and plasma cells. Apart from their known function at the forefront in secreting protective antibodies, plasma cells have recently been appreciated for their role in many biological functions through cytokines production [23–28]. Furthermore, although circulating antibodies are mainly produced by long-lived plasma cells, some reports suggest that short-lived plasmablasts could also persist and participate in maintaining antigen-specific circulating antibodies levels [29, 30]. Similar to plasma cells, activation of plasmablasts isolated from human PBMCs has been shown to result in secretion of high levels of IL-6 and induction of follicular helper T cells [31]. Therefore, we investigated these non-canonical antibody-independent functions of different B-cell subsets, which could contribute to host-pathogen interactions. Our results showed that B-cell subsets could participate in these antibody-independent functions by secreting pro-inflammatory cytokines in MERS-recovered individuals upon specific antigen stimulation. Nonetheless, further studies are clearly needed to better understand the role of these functions.

Interestingly, we observed high level of IL-17 expression from memory $CD8^+$ T cells upon ex vivo restimulation from MERS recovered individuals but not healthy donors (Figure 4) in which the levels of IL-17 expression were higher than IFN-γ and TNF-α. IL-17 is a known pro-inflammatory cytokine, and its expression is usually linked with Th17 $CD4^+$ T cells. Nonetheless, accumulating body of evidence shows that other immune cells could also express IL-17 including memory $CD8^+$ T cells (ie, Tc17 cells) in humans and animals in response to infection, vaccines, and inflammatory disease [32, 33]. These memory Tc17 cells represent a distinct non-cytotoxic subset from type 1 $CD8^+$ T (Tc1) cells which mainly produce IFN-γ and associated with high cytotoxic capacity. They also show persistence and production of multiple cytokines including IFN-γ and TNF-α among others [33, 34]. Although Tc17 cells involvement in immune-mediated pathology has been suggested to be mediated via pro-inflammatory cytokine production not cytotoxic mechanism [32, 35], its role in MERS-CoV and other viral infections merits further investigations especially that several reports have shown association between increased levels of IL-17 and immune-mediated pathology, disease severity, and increased mortality in MERS-CoV and other viral infections [36–38].

As vaccines are currently not available for MERS-CoV which is still endemic for more than 8 years now in Saudi Arabia and surrounding countries, a better understanding of the longevity and quality of the immune response is needed. Interestingly, reinfection with MERS-CoV in humans has not been reported, which could be a sign of development of protective immunity after the first infection. Indeed, current and previous reports suggest that robust and persistent nAbs and memory B and T cells responses after a single infection with MERS-CoV could provide a prolong protection from reinfection. However, this need to be further examined either by following a larger cohort for long time or experimentally in animal models. Whether such immunity can also protect from other related betacoronaviruses such as SARS-CoV-2 is currently unknown; however, we didn't find any cross reactivity of our cohort serum samples with SARS-CoV-S or N antigens by ELISA (data nor shown). Furthermore, because effective therapeutics options to treat MERS patients are lacking, passive antibody therapy using plasma from convalescent individuals have been suggested, and previous studies reported a reduction in viral loads and an improvement in clinical symptoms in patients infected with different CoVs [39, 40]. Our results support the notion that convalescent plasma, especially from those with high nAb titers, could be a potential therapy for the treatment of MERS infections.

Comprehensive understanding of specific T- and memory B-cell responses for highly pathogenic CoVs are lacking. As SARS-CoV-2-specific T and B cells have just started to be characterized, understanding of such responses and their potential protective role can be inferred from studies of patients who recovered from SARS-CoV-1 and MERS-CoV. Our study shows that recovery from MERS-CoV is associated with detectable and persistent MERS-specific humoral and cellular memory responses that can last for up to 6.9 years, which could provide a prolong protective immunity. These data advance our understanding of the immunity to MERS-CoV and likely other human betacoronaviruses, which is relevant to the ongoing COVID-19 pandemic.

Limitations of the Study

The results of our analysis cannot completely exclude the possibility of cross-reactive antibodies, B cells, or T cells against other seasonal coronaviruses contributing to the high levels observed in our study. We also cannot fully rule out potential mild or infections with SARS-CoV-2 especially that these samples were collected in early 2020. However, our assays were mainly based on S1 or RBD antigens, which tend to be more divergent

than other proteins such as N. Also, we could not detect any cross reactivity of our cohort serum samples with SARS-CoV-S or N antigens. Therefore, this seems unlikely, especially that previous reports have shown strong cellular responses in MERS patients. Furthermore, a lack of serial samples and the small number of cases included in this study especially in the analysis of B and T cells are major limitations, thus future studies should include larger cohorts and longitudinal sampling.

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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Author contributions. R. A. A., A. A., A. B. M., A. A. A., W. A., and A. M. H. were in charge of conceptualization, study design, and supervision of the work. R. A. A., I .S. A., A. M. A., O. A. A.-G., Y. A. A., H. M. A., and M. M. A. collected data and provided samples. R. Y. A., A. B. M., A. A. A., S. A.-A., M. B., M. A. A., and W. B. performed experiments and data analysis. A. B., Z. A. M., W. A., and A. M. H. provided resources. R. A. A. and A. M. H. conducted data analysis and generated figures. R. A. A., A. A., A. B. M., A. A. A., and A. M. H. contributed to the writing of the first manuscript draft. Manuscript was reviewed, edited, and approved by all authors.

Potential conflicts of interest. A. M. H. reports consultation fees from SaudiVax ltd and US10849972B2 patent (Trimeric S1-CD40L fusion protein vaccine against Middle East respiratory syndrome-coronavirus), US10806784B1 patent (composition and method for treating MERS), US11154612B2 patent (MERS-CoV vaccine with trimeric S1-CD40L fusion protein), US11219685B1 patent (Intranasal MERS-CoV vaccine), US20220008530A1 patent application (MERS-CoV VACCINE COMPOSITION), US20220054629A1 patent application (nucleic acid based vaccine against middle east respiratory syndrome-coronavirus). All other authors report no potential conflicts. 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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