Supplementary Materials

MERS-CoV infection elicits long-lasting specific antibody, T and B cell immune responses in recovered individuals

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Supplementary Materials and Methods

Clinical Samples
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. All serum samples were collected after centrifugation of blood samples at 3500 rpm for 5 min and frozen at –80°C for serological analysis. All serum samples were heat-inactivated at 56°C for 30 min before their use. Peripheral blood mononuclear cells (PBMCs) were obtained from 10 MERS recovered individuals and 4 healthy controls. The PBMCs were isolated using Lymphosep™ (MP Biomedicals, Irvine, CA) according to manufacturer protocols, and cryopreserved in freezing medium at -80°C. Sample collection was performed in accordance with the human ethical approval from Directorate of Health Affairs, Saudi Ministry of Health (Project number 1496). Written or verbal informed consents were obtained from all participants.

Recombinant proteins
Recombinant MERS-CoV spike S1 subunit (amino acids 1–725) and receptor binding domain (RBD) (amino acids 367-606), all expressed and purified from HEK293 Cells, were purchased commercially (Sino Biological, China). Recombinant MERS-CoV nucleocapsid (N) protein was expressed and purified in-house from Escherichia coli BL21 (DE3) cells using a nickel-nitrilotriacetic acid (Ni-NTA) column as previously described [1].

Cells
African Green monkey kidney-derived Vero E6 cells (ATCC 1586) and Baby Hamster kidney BHK-21/WI-2 cell line (Kerafast, Boston, MA) were cultured in Dulbecco’s modified essential medium (DMEM) supplemented with 100 U/mL of penicillin, 100 μg/mL of streptomycin and 5% or 10% FBS at 37°C, 5% CO₂.

Detection of MERS-CoV binding IgG by ELISA
Detection of anti-N, -S1 and -RBD IgG antibodies against MERS-CoV was performed as previously described [1,2]. Briefly, 96-well high binding ELISA plates (Greiner Bio One, Monroe, NC) coated with each individual recombinant proteins (i.e. S1 (1 μg/ml), RBD (1 μg/ml) or N (4
μg/ml) protein) were washed with phosphate-buffered saline (PBS) containing 0.05% tween-20 (PBS-T) and blocked with 5% skim milk in PBS-T (blocking buffer) at 37°C for 1 hour. After washing, serially diluted serum samples starting from 1:100 dilution in blocking buffer were added to wells and incubated for 1 hour at 37°C. Plates were then washed and incubated with HRP-conjugated goat anti-human IgG (H + L) (Jackson ImmunoResearch, West Grove, PA) for 1 hour, washed again, and incubated with 3,3′,5,5′- tetramethylbenzidine (TMB) substrate (KPL, Gaithersburg, MD) at room temperature for 30 minutes. The reaction was stopped using 0.16 M sulfuric acid, and absorbance was measured at 450 nm on a Synergy 2 Multi-Detection Microplate Reader (BioTek, Winooski, VT). Half-maximal binding (EC$_{50}$) values were calculated using GraphPad Prism V9 software (GraphPad Co., San Diego, CA).

**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 [3]. Briefly, BHK21/W1-2 cells transfected with pcDNA expressing codon-optimized truncated S protein (lacking the last 21 residues at the C-terminal) from MERS-CoV (GenBank accession number: KF958702) were infected with rVSV-ΔG/G*-luciferase (Kerafast, Boston, MA) 24 hours later. After washing, cells were incubated in DMEM containing rabbit polyclonal anti VSV-G antibody for 24 hours at 37°C and 5% CO$_2$, and supernatant containing rVSV-ΔG/MERS-S*-luciferase pseudovirus was then collected 24 hours later. For neutralization assay, serially diluted serum sample starting from 1:10 dilution in DMEM with 5% FBS were mixed with equal volume of media containing rVSV-ΔG/MERS-S*-luciferase pseudovirus that yields 1 × 10$^5$ RLU and incubated in a 5% CO$_2$ humidified incubator at 37°C for 1 hour in duplicates. Then, 100 μl of the mixture were transferred onto Vero E6 cell monolayers in 96-well white plate with clear bottom (COSTAR) and incubated at 37°C and 5% CO$_2$ for 24 hours. This was followed by cell lysis and measuring luciferase activity as a relative luciferase unit (RLU) using the Luciferase Assay System (Promega, Madison, WI) according to the manufacturer’s instructions. Cell only control (CC) and virus control (VC) were included in each run. The inhibition of luciferase activity by each dilution of the serum sample was determined as follows: 100 – [(average RLU from each dilution – average RLU from CC) / (average RLU from VC – average RLU from CC) × 100]. Then, neutralization titers were calculated as median
inhibitory concentration (IC$_{50}$) using four-parameter logistic (4PL) curve in GraphPad Prism V9 software (GraphPad Co., San Diego, CA).

**Antigen-specific memory B cells detection**

To detect antigen specific B cells, MERS-CoV S1 protein was biotinylated for 1 hour at 4°C according to the manufacturer instructions (Abcam, UK). Biotinylated MERS-CoV S protein was then multimerized with phycoerythrin labeled streptavidin (SA-PE) at 2:1 ratio (~4:1 molar ratio). SA-PE was used in the absence of the biotinylated MERS-CoV S to gate out MERS-CoV S1 non-specific binding cells. For staining, 1 million cryopreserved PBMCs were plated in a 96-well plate and blocked for 15 mins. After washing the cells with FACS buffer (PBS with 2% heat inactivated FBS), the cells were stained with 25ng MERS-S1-PE for 1 hour at 4°C. Cells were then washed and incubated for 30 mins at 4°C with BV510-conjugated anti-CD19 (Clone HIB19), PE-Cy7-conjugated anti-CD27 (Clone O323), APC-conjugated anti-CD38 (Clone HB-7), PerCP-Cy5.5-conjugated anti-CD138 (Clone DL-101), PB-conjugated anti-IgD (Clone IA6-2), and Alexa Fluor® 700-conjugated anti-IgM (Clone MHM-88) antibodies. Next, the cells were washed, fixed, and acquired using BD FACSArray™ III flow cytometer. All data were analyzed using FlowJo v10 software (Tree Star Inc., Ashland, OR). All antibodies used were from BioLegend, UK.

**Antigen-specific memory B and T cells response detection**

One million cryopreserved PBMCs/well were re-stimulated with either recombinant S1 protein at a concentration of 1µg/ml to activate antigen-specific B cells or with 5 µg/ml of a pool of 15-mer peptides overlapping by 11 amino acid and covering the entire MERS-CoV S protein (GenScript USA Inc, Piscataway, NJ, USA) to activate antigen-specific T cells. Re-stimulation was done for 16 hours at 37°C in the presence of brefeldin A (BD Biosciences, San Jose, CA) at a final concentration of 1:1000. Cells stimulated with phorbol myristate acetate/ionomycin were used as a positive control, and cells in RPMI 1640 medium were used as a negative unstimulated control. Cells were then washed in FACS buffer and stained with LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit, for 633 or 635 nm excitation (Invitrogen, Carlsbad, CA) for 30 min at room temperature. Cells were then washed and incubated for 20 mins at 4°C with BV510-conjugated anti-CD19 (Clone HIB19), PE-Cy7-conjugated anti-CD27 (Clone O323), APC-conjugated anti-CD38 (Clone HB-7), PE-CF594-conjugated anti-CD24 (Clone ML5) and PerCP-Cy5.5-
conjugated anti-CD138 (Clone DL-101) surface antibodies to stain B cells, while T cells were stained with APC-Cy7-conjugated anti-CD3 (Clone HIT3a), BV510-conjugated anti-CD4 (clone OKT4), PE-Cy7-conjugated anti-CD8 (clone SK1), PerCP-Cy5.5-conjugated anti-CD45RA (clone HI100) and APC-conjugated anti-CCR7 (clone G043H7) surface antibodies. Cells were then washed with FACS buffer and fixed and permeabilized using Cytofix/Cytoperm Solution (BD Biosciences, San Jose, CA) according to the manufacturer’s protocol. For intracellular staining, cells were incubated for 20 mins at 4°C with PE-conjugated anti-IL-6 antibody (Clone MQ2-13A5), PE–conjugated anti-IL17α antibody (clone BL168), FITC-conjugated anti-IFN-γ antibody (clone B27) and BV421-conjugated anti-TNF-α antibody (clone Mab1K). After washing the cells twice with permeabilization buffer, cells were fixed with 4% paraformaldehyde and acquired using BD FACSARia™ III flow cytometer. All data were analyzed using FlowJo v10 software (Tree Star Inc., Ashland, OR). All antibodies used were from BioLegend, UK.

Statistical analysis
Comparisons between groups were performed using T-test. Analyses were performed using GraphPad Prism V9 software (GraphPad Co., San Diego, CA).
Reference

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2. Al-Amri SS, Hashem AM. Qualitative and Quantitative Determination of MERS-CoV S1-Specific Antibodies Using ELISA. Methods Mol Biol, 2020; 2099, 127–133.

3. Almahboub SA, Algaissi A, Alfaleh MA, ElAssouli MZ, Hashem AM. Evaluation of Neutralizing Antibodies Against Highly Pathogenic Coronaviruses: A Detailed Protocol for a Rapid Evaluation of Neutralizing Antibodies Using Vesicular Stomatitis Virus Pseudovirus-Based Assay. Front Microbiol, 2020; 11: 2020.
Supplementary Figures
Supplementary Fig. S1. Gating strategy of memory B cells. PBMCs were isolated from MERS recovered individuals and healthy donors, and (a) incubated with MERS-S1 conjugated to PE or (b) restimulated with MERS-S1 protein. Figure shows the gating strategy on live CD19+ B cell subpopulations including (a) MERS-S1-specific non-class switched B cells (CD19+IgD+IgM+), class-switched memory B cells (MBC) (CD19+CD27+IgD-IgM-CM38-); and (b) TNF-α, IL-6 and IFN-γ producing memory B cells (CD19+CD38-CD27+CD24+), plasma B cells (CD19+CD38+CD27+CD24-CD138+) and plasmablast B cells (CD19+CD38+CD27+CD24-CD138-).
Supplementary Fig. S2. Gating strategy of memory T cells. PBMCs were isolated from MERS recovered individuals and healthy donors, and ex vivo re-stimulated with MERS-CoV S overlapping peptide pool. Figure shows the gating strategy on live CD3+CD8+ and CD3+CD4+ T cell subpopulations including CD8+CCR7+CD45RA− central memory cells (Tcm), CD8+CCR7−CD45RA− effector memory cells (Tem) and CD8+CCR7−CD45RA+ terminally differentiated effector cells (TEMRA), in addition to CD4+CCR7+CD45RA− central memory cells (Tcm) and CD4+CCR7−CD45RA− effector memory cells (Tem).
Supplementary Fig. S3. Single-, double- and triple-cytokine producing memory T cells. Representative FACS plots of single-, double- and triple-cytokine–producing cells in stimulated and non-stimulated (a) central memory CD8+ T cells (CD3+CD8+CD197+CD45RA-), (b) effector memory CD8+ T cells (CD3+CD8+CD197CD45RA-), (c) terminally differentiated effector CD8+ T cells (CD3+CD8+CD197CD45RA-), (d) central memory CD4+ T cells (CD3+CD4+CD197 +CD45RA-) and (e) effector memory CD4+ T cells (CD3+CD4+CD197CD45RA-) from MERS recovered (R) individuals and healthy donors (H).
Supplementary Table. Average percentage of antigen-specific B and T cell subsets from total lymphocytes population.

|                          | MERS-S1 specific cells | Class switched memory B cells |
|--------------------------|------------------------|------------------------------|
|                          | 0.007                  | 0.045                        |

|                        | B cells                 |                                        |
|------------------------|-------------------------|----------------------------------------|
|                        | Non-class switched cells | Class switched memory B cells          |
|                        |                         |                                        |
|                        | Memory B cells          | Plasmablast B cells                   | Plasma cells |
|                        |                         |                                        |              |
| TNF-α                  | 0.010                   | 0.006                                  | 0.026        |
| IL-6                   | 0.008                   | 0.005                                  | 0.030        |
| IFN-γ                  | 0.006                   | 0.002                                  | 0.011        |

|                        | CD8+ T cells             |                                        |
|                        |                         |                                        |
|                        | Central memory cells     | Effector memory cells                  | TEMRA        |
|                        |                         |                                        |              |
| TNF-α                  | 0.001                   | 0.002                                  | 0.001        |
| IL-17                  | 0.021                   | 0.011                                  | 0.021        |
| IFN-γ                  | 0.009                   | 0.002                                  | 0.001        |

|                        | CD4+ T cells             |                                        |
|                        |                         |                                        |
|                        | Central memory cells     | Effector memory cells                  |
|                        |                         |                                        |
| TNF-α                  | 0.016                   | 0.005                                  |
| IL-17                  | 0.002                   | 0.002                                  |
| IFN-γ                  | 0.005                   | 0.003                                  |