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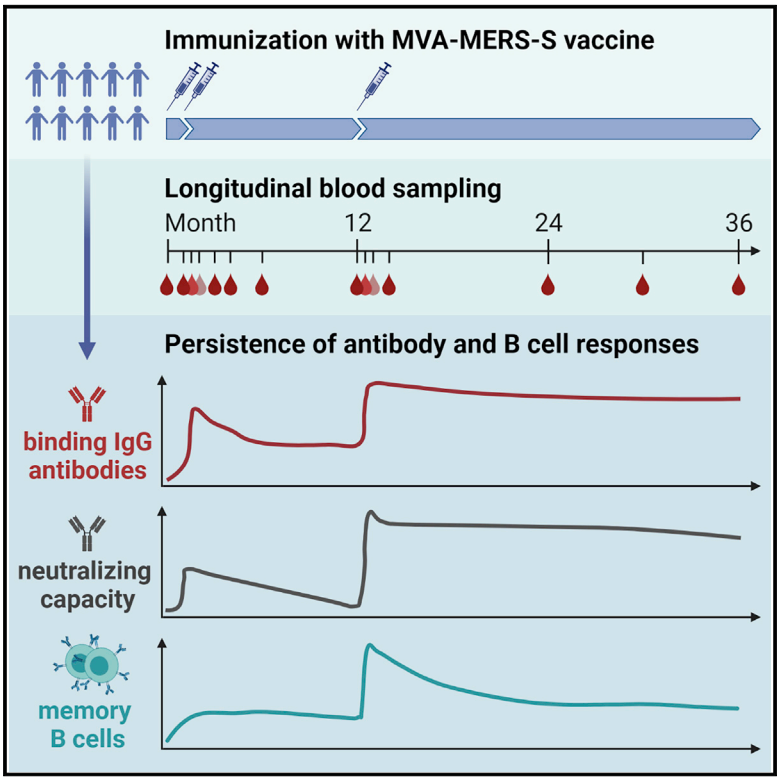
Persistence of MERS-CoV-spike-specific B cells and antibodies after late third immunization with the MVA-MERS-S vaccine

Graphical abstract



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In brief

Weskamm et al. longitudinally describe B and T cell responses as well as antibody subclasses and neutralization capacity induced by three homologous immunizations with the MVA-MERS-S vaccine candidate. A late booster vaccination significantly enhances the frequency and persistence of memory B cells, binding IgG1 and neutralizing antibodies.

Highlights

- A late boost with the MVA-MERS-S vaccine enhances frequency of antibodies and B cells
- Binding IgG and neutralizing antibodies persist for 2 years after the late boost
- Responses specific to MERS-CoV-spike S1 and S2 subunits show distinct dynamics
- Vaccine-induced antibodies predominantly belong to IgG1 and IgG3 subclasses

Weskamm et al., 2022, Cell Reports Medicine *3*, 100685
July 19, 2022
https://doi.org/10.1016/j.xcrm.2022.100685

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Persistence of MERS-CoV-spike-specific B cells and antibodies after late third immunization with the MVA-MERS-S vaccine

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https://doi.org/10.1016/j.xcrm.2022.100685

SUMMARY

The Middle East respiratory syndrome (MERS) is a respiratory disease caused by MERS coronavirus (MERS-CoV). In follow up to a phase 1 trial, we perform a longitudinal analysis of immune responses following immunization with the modified vaccinia virus Ankara (MVA)-based vaccine MVA-MERS-S encoding the MERS-CoV-spike protein. Three homologous immunizations were administered on days 0 and 28 with a late booster vaccination at 12 ± 4 months. Antibody isotypes, subclasses, and neutralization capacity as well as T and B cell responses were monitored over a period of 3 years using standard and bead-based enzyme-linked immunosorbent assay (ELISA), 50% plaque-reduction neutralization test (PRNT50), enzyme-linked immunospot (ELISpot), and flow cytometry. The late booster immunization significantly increases the frequency and persistence of spike-specific B cells, binding immunoglobulin G1 (IgG1) and neutralizing antibodies but not T cell responses. Our data highlight the potential of a late boost to enhance long-term antibody and B cell immunity against MERS-CoV. Our findings on the MVA-MERS-S vaccine may be of relevance for coronavirus 2019 (COVID-19) vaccination strategies.

INTRODUCTION

The Middle East respiratory syndrome (MERS) is a viral respiratory disease caused by MERS-coronavirus (MERS-CoV),[1,2] which was first identified in a patient in Saudi Arabia in 2012.[3] As a disease of the lower respiratory tract, MERS can progress rapidly from unspecific, influenza-like symptoms to severe pneumonia, multiple organ failure, and death. Primary transmission of MERS-CoV infections is linked to exposure to dromedary camels.[4,5] Secondary infections can occur via human-to-human transmission, with nosocomial and household outbreaks accounting for most cases.[6,7] As of February 2022, 2,585 cases, including 890 associated deaths, were reported in 27 countries with a case fatality rate of 34.4%.[8] There are no licensed vaccines or specific therapeutic options available to prevent or treat MERS-CoV infection. However, effective countermeasures are crucial to avoid potential future outbreaks and to prevent infections of persons at risk.

In general, vaccine-induced immune responses include activation and priming of naïve T and B cells. With regard to the B cell population, vaccination induces short living plasmablasts and plasma cells secreting high amounts of antibodies. Furthermore, B cell activation can result in the generation of two long-lasting cell populations, namely long-lived plasma cells (LLPCs) and memory B cells (MBCs). Both can be maintained for a long time in the absence of the specific antigen[9,10] and contribute to the maintenance of immunological memory. Antibodies can exhibit neutralizing activity via their fragment

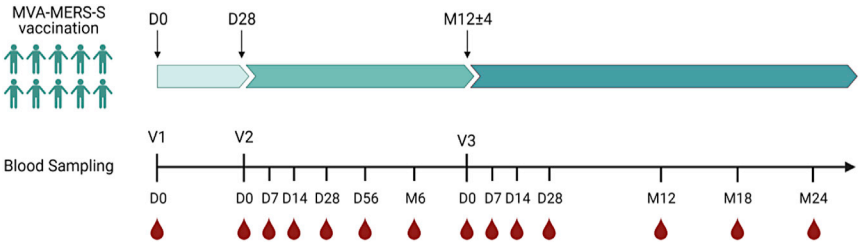


Figure 1. Study design

Study participants (n = 10) received two vaccinations wit MVA-MERS-S (V1 and V2) 28 days apart and a late third vaccination (V3) at 12 ± 4 months after prime. Blood was frequently sampled for up to 2 years after V3 at indicated time points (see also Table S1). Figure was created with BioRender.com.

antigen-binding (Fab) region, whereas structural properties of the fragment crystallizable (Fc) region classify antibodies into immunoglobulin (Ig) isotypes (e.g., IgA, IgG, IgM) and subclasses (IgA1–2, IgG1–4) that differ in their abundance and non-neutralizing functional capacities. While the exact vaccine-induced correlates of protection against MERS have not been identified thus far, humoral immune responses are considered to be critical in mediating protection against infection and severe disease.

A variety of MERS vaccines have been developed and tested in preclinical stages.[2,11] Most of them are based on the MERS-CoV-spike (S) protein, which is the primary inducer of the host immune response. MERS-CoV-S is composed of an N-terminal S1 subunit and a C-terminal S2 subunit and can be cleaved at the junction between the two subunits either during viral biogenesis or upon encounter of target cells.[12,13] The S protein acquires different conformations while mediating the entry of the virus into the host cell. To initiate infection, the receptor-binding domain (RBD) contained in the S1 subunit recognizes its host cell receptor dipeptidylpeptidase 4 (DPP4), followed by the fusion of viral and host cell membrane mediated by the S2 subunit.[14,15] Neutralizing antibodies that target the S protein can therefore block virus attachment and entry into the host cell. Thus far, only three vaccine candidates have reported their results from early-phase clinical trials showing safety and immunogenicity.[16–19] One of them is the viral vector vaccine MVA-MERS-S, which is based on the recombinant modified vaccinia virus Ankara (rMVA) vector platform and encodes the full-length S protein (GenBank: JX869059).[20] Between 2017 and 2019, we evaluated MVA-MERS-S at two dose levels (low dose: 1×10^7 plaque-forming units [PFUs], high dose: 1×10^8 PFU) for safety and immunogenicity in a first-in-human phase 1 clinical trial in 23 participants[16] (ClinicalTrials.gov: NCT03615911).

A homologous prime-boost immunization using a 28-day interval revealed a benign safety profile and was effective in inducing humoral and cell-mediated immune responses against MERS-CoV-S1.[16] Seroconversion was detected in 87% of all participants and in 100% of the high-dose cohort. However, antibodies waned within 6 months. A follow-up study was initiated to assess safety and immunogenicity of a late third immunization with MVA-MERS-S (1×10^8 PFU) at 12 ± 4 months after prime vaccination in a subgroup of 10 participants.[21] Seven of these individuals were included in a long-term observational study and followed up with for another 2 years.

We here analyzed antibody as well as T and B cell responses specific to the S1 and S2 subunit of MERS-CoV-S to gain comprehensive insights into adaptive immunity induced by three vaccinations with MVA-MERS-S. A specific focus of our study is the induction and longevity of MBCs as well as the distribution of antibody isotypes and subclasses. Antibody analysis was conducted using a standard and a bead-based multiplex enzyme-linked immunosorbent assay (ELISA) as well as a 50% plaque-reduction neutralization test (PRNT50), whereas T and B cell responses were assessed by enzyme-linked immunospot (ELISpot) and flow cytometry assays. Our findings highlight the benefits of a late third vaccination, demonstrating for the first-time persistence of MERS-CoV-specific vaccine-induced humoral immunity over a 2-year period.

RESULTS

Being part of a phase 1 trial initiated in December 2017, a subgroup of ten study participants received three doses of the viral vector vaccine MVA-MERS-S encoding the MERS-CoV-S protein. The first two immunizations were administered 28 days apart with a late third immunization 12 ± 4 months after prime vaccination. Immune responses were monitored after each vaccination and followed up on for another 2 years (Figure 1).

Late third immunization with MVA-MERS-S resulted in robust and long-lasting binding IgG and neutralizing antibodies but less pronounced T cell responses

In a first step, we monitored MERS-CoV-S-specific antibodies and T cells to gain a broad overview on adaptive immune responses following three vaccinations with MVA-MERS-S (Figure 2).

Binding IgG antibody responses were evaluated using an in-house ELISA based on a full-length recombinant MERS-CoV-S protein (Figure 2A). Both the second and third immunization (V2 and V3, respectively) induced S-specific IgG antibody levels above the cutoff in all ten vaccinees. The IgG responses induced by V2 peaked at V2:day 14 and declined during the next months but stayed above the cutoff (optical density [OD] 0.1) in 100% of the study participants (n = 10/10) for approximately 12 months after prime immunization (V3:day 0). Following V3, antibody levels rapidly increased and were maintained above cutoff throughout the entire study period for all analyzed vaccinees (n = 7/7; V3:months 18–24). IgG responses induced by V2 reached a median OD of 0.8 at V2:day 14 and showed a broad range in magnitude (min–max: 0.3–1.8 OD). In comparison, V3 induced a very strong and homogenous response of S-specific IgG in all vaccinees, resulting in a median OD value of 1.8 (min–max: 1.6–2.3 OD) and a fold change of 2.6 at V3:day 14 compared with V2:day 14.

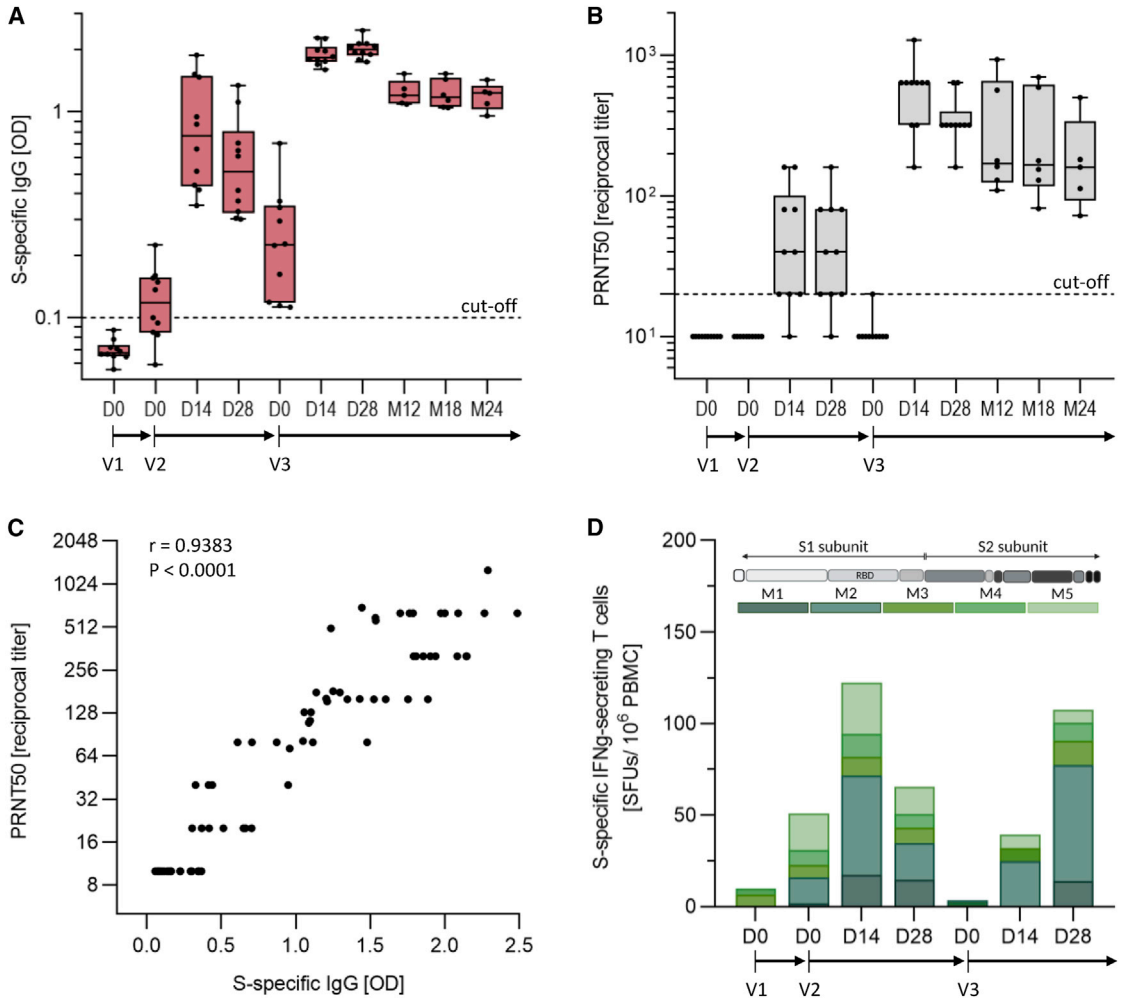


Figure 2. MERS-CoV-S-specific humoral and T cell responses induced by three vaccinations with MVA-MERS-S

(A) Longitudinal dynamics of S-specific IgG antibodies. Shown are the optical density (OD) values measured at 450–620 nm by ELISA. Data are represented as individual data points (mean of technical duplicates) and median ± interquartile range (IQR).

(B) Neutralization activity of serum antibodies as measured by 50% plaque-reduction neutralization test (PRNT50). Data are represented as individual data points and median ± IQR.

(C) Spearman correlation between S-specific IgG antibodies and serum neutralization activity.

(D) T cell responses as measured by IFNγ ELISpot after stimulation with five overlapping peptide (OLP) pools (M1–M5), spanning the entire MERS-CoV-S amino acid sequence. Shown are the median values of spot forming units (SFUs; mean of technical triplicates) across all vaccinees (n = 10) for each OLP pool. Number of samples, median, and p values for each time point and all three assays are shown in Table S2.

Serum neutralization capacity was assessed by PRNT50 (Figure 2B). While V1 did not induce neutralizing antibodies, V2 led to an increased titer in 90% of participants (n = 9/10) with a median reciprocal titer of 40 (min–max: 10–160) at V2:day 14. Neutralizing antibody titers declined during the following year, resulting in titers below the cutoff in 90% (n = 9/10) of the study participants. Upon V3, all participants generated neutralizing antibody responses, showing a strong increase compared with V2 with a median reciprocal titer of 640 (min–max: 160–1,280) at V3:day 14. During the following 12–24 months, the titer of neutralizing antibodies continuously decreased but stayed above the threshold in 100% of the analyzed study participants (n = 7/7). Two years after the late boost immunization, 80% of the participants (n = 4/5) showed neutralizing antibody titers persisting at levels at least 3-fold higher (min–max: 3- to 16-fold) compared with the peak response induced by the second vaccination (V2:day 14), whereas one vaccinee showed a titer similar to V2:day 14.

S-specific IgG antibody titers measured by ELISA showed a positive correlation with the serum neutralization capacity measured by PRNT50, as shown in Figure 2C ($r = 0.9383$, $p < 0.0001$).

Besides antibodies, T cell responses also represent a key element of the adaptive immune system and were evaluated by interferon-γ (IFNγ) ELISpot in this study. Peripheral blood mononuclear cells (PBMCs) were stimulated with five

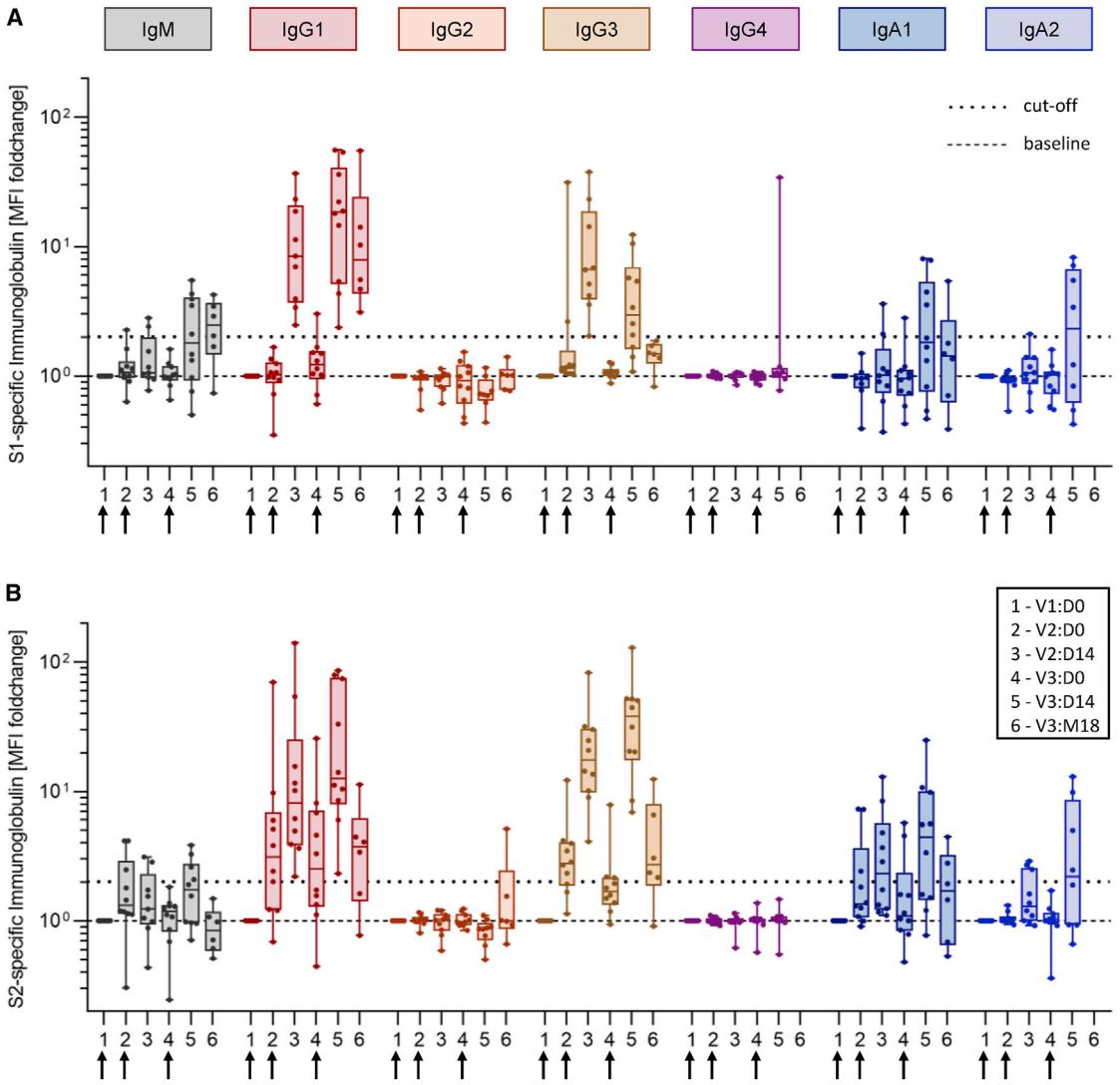


Figure 3. Isotype and subclass distribution within vaccine-induced MERS-CoV-S-specific antibodies

S1- (A) and S2- (B) specific responses of IgM, IgG1–4, and IgA1–2 at different time points after vaccination, displayed as fold changes of median fluorescence intensities (MFIs; measured by bead-based ELISA) compared with baseline values at V1:day 0. Data are represented as individual data points (mean of technical duplicates) and median ± IQR. Number of samples and median fold changes are shown in Table S3.

overlapping peptide (OLP) pools (M1–M5) spanning the entire MERS-CoV-S protein. S-specific T cell responses first emerged after a single shot of MVA-MERS-S in 40% of the vaccinees (n = 4/10; V2:day 0) and were further enhanced after the V2, with peak responses observed at V2:day 14 (Figure 2D; see also Figure S1 and Koch et al.[16]). Overall, MERS-CoV-S-specific IFNγ secretion was detected in 80% (8/10) of all participants at one or more time points throughout the first two vaccinations until V3:day 0. Following the third vaccination, 50% of the participants (n = 5/10) showed an IFNγ secretion at the analyzed time points. Across all study participants, T cell responses were observed to all five OLP pools, but responses to pool M2, which covers the RBD sequence, were most frequently detected (n = 7/10). Depicting the median value of IFNγ responses across all ten vaccinees for each OLP pool, Figure 2D demonstrates the peak response at V2:day 14 as well as the predominant responses to M2 after both V2 and V3.

Taken together, these data demonstrate that V2 induced detectable humoral and T cell responses in 100% (n = 10/10) and 80% (n = 8/10) of vaccinees, respectively, which declined during the following months. The late V3 homogenously induced anti-S IgG levels in 100% (n = 10/10) of the vaccinees, exceeding the levels induced by V2 and showing enhanced persistence. In comparison, T cell responses after V3 were more diverse and less pronounced than after V2.

Antibodies induced by MVA-MERS-S predominantly belong to IgG1 and IgG3 subclasses

To gain insight into the distribution of antibody isotypes and subclasses induced by MVA-MERS-S vaccination, we longitudinally

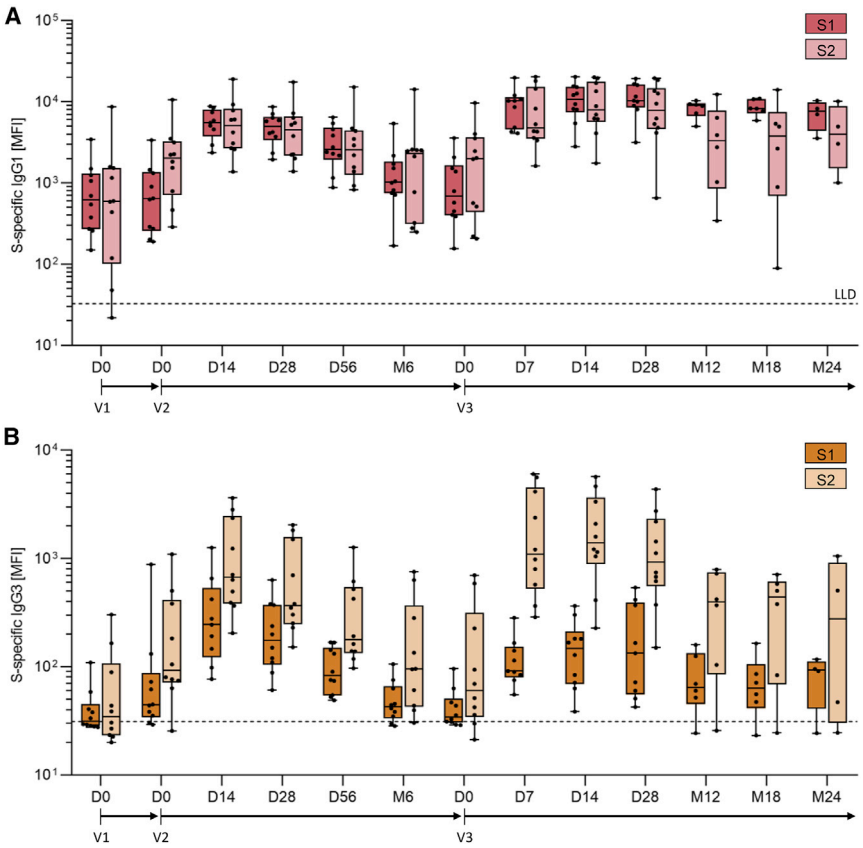


Figure 4. Longitudinal dynamics of MERS-CoV-S-specific IgG1 and IgG3 antibodies

Vaccine-induced S1/S2-specific IgG1 (A) and IgG3 (B) are displayed as MFI, measured by bead-based multiplex ELISA. Data are represented as individual data points (mean of technical duplicates) and median ± IQR. LLD, lower limit of detection. Number of samples, median, and p values for each time point are shown in Table S4.

analyzed plasma samples using a bead-based multiplex ELISA. The median fluorescence intensity (MFI) was used as a relative measure for antibody responses. An induction of 2-fold above baseline (V1:day 0) was defined as a positive assay response. For IgM and IgA antibodies, we observed only a slight induction that was not consistent throughout the cohort (Figures 3A and 3B). With regard to IgG subclasses, MERS-CoV-S-specific IgG1 and IgG3 showed vaccine-induced responses with peak levels observed 14 days after V2 and V3. By contrast, only one of the participants reached the threshold values for IgG2 and IgG4, respectively (Figures 3A and 3B).

At V2:day 14, 100% of the study participants showed a positive assay response for S1- and S2-specific IgG1 and IgG3 (S1: n = 9/9; S2: n = 10/10). Plasma levels of both subclasses waned during the following months until V3:day 0, resulting in levels above cutoff in 10% (S1/IgG1, n = 1/10), 0% (S1/IgG3, n = 0/10), 50% (S2/IgG1, n = 5/10), and 30% (S2/IgG3, n = 3/10) of the study participants, respectively. V3 reinduced S1-specific IgG1 and S2-specific IgG1 and IgG3 in 100% (n = 10/10) as well as S1-specific IgG3 in 70% (n = 7/10) of participants. Notably, the antibody responses induced by V3 persisted for a longer period of time than those induced by V2. At the late time point V3:month 18, antibody levels persisted above the cutoff in 100% (S1/IgG1, n = 6/6), 0% (S1/IgG3, n = 0/6), 66.7% (S2/IgG1, n = 4/6), and 83.3% (S2/IgG3, n = 5/6) of participants.

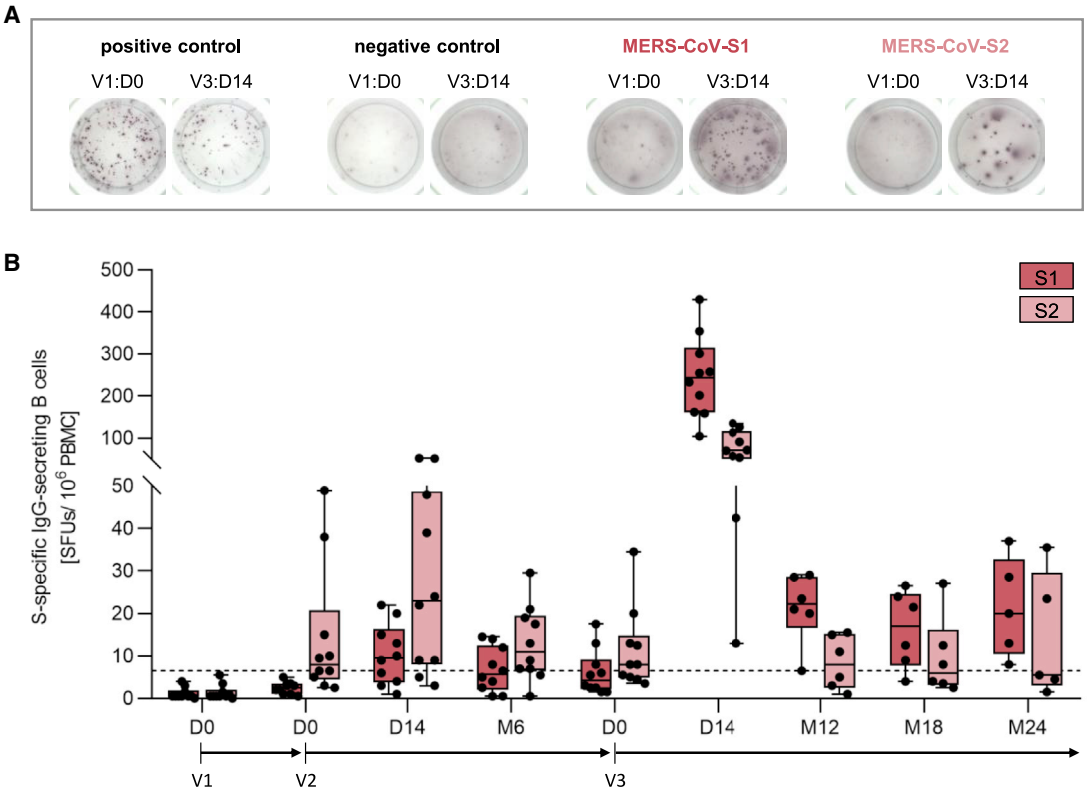
S2-specific antibodies were induced earlier than those specific to S1, with S2-specific IgG1 and IgG3 responses detected 28 days after prime at V2:day 0 in 70% (n = 7/10) of the study participants. In comparison, S1-specific IgG3 was only detectable in 20% (n = 2/10) of the participants, and no vaccinee showed S1-specific IgG1 at this early time point (Figures 3A and 3B).

Individual levels of S1/S2-specific IgG1 and IgG3 are depicted in Figures 4A and 4B, highlighting that IgG1 was homogeneously and strongly induced in all participants after both V2 and V3, while the magnitude of IgG3 responses showed a broader distribution within the cohort. Comparing peak responses induced by V2 and V3, S1-specific IgG1 showed a 2.3-fold increase after V3 compared with V2. S2-specific responses resulted in a fold change of 1.7. Comparing the median of MFI values, S1- and S2-specific IgG1 reached similar levels at V2:day 14 (S1: 5,483; S2: 5,079), whereas S1- exceeded S2-specific IgG1 at V3:day 14 (S1: 10,714; S2: 7,894). In comparison, S1-specific IgG3 responses decreased at V3:day 14 compared with V2:day 14, whereas IgG3 responses toward S2 increased 1.6-fold. As opposed to IgG1, IgG3 responses resulted in higher MFIs for S2- than for S1-specific responses both after V2 (S1: 244; S2: 671) and V3 (S1: 147; S2: 1,401). Although peak responses after V3 were observed at V3:day 14, S1- and S2-specific IgG1 and IgG3 were already elevated at V3:day 7, indicating a rapid response of antibody secretion following the late immunization. The peak antibody level was prolonged until V3:day 28.

MERS-CoV-S-specific B cells were induced by MVA-MERS-S vaccination and persisted for up to 2 years

We here investigated the frequency of antigen-specific B cells (ASBCs) induced by MVA-MERS-S vaccination using an IgG ELISpot with pre-stimulated PBMCs. MERS-CoV-S1/S2-specific ASBCs were quantified as spot-forming units (SFUs) per million PBMCs (Figures 5A–5C). ASBCs showed a first peak 14 days after the second vaccination (V2:day 14), but their numbers declined during the following 8–14 months. Notably, they were again rapidly induced after V3 at numbers significantly higher than after V2.

Comparing responses against the two MERS-CoV-S subunits, S2-specific ASBCs were already induced by V1 in 50% of vaccinees (V2:day 0: n = 5/10, median SFU = 8), while S1-specific ASBCs were not detectable at this early time point. V2 induced



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Time point	V1:D0	V2:D0	V2:D14	V2:M6	V3:D0	V3:D14	V3:M12	V3:M18	V3:M24
n	10	10	10	10	10	10	6	6	5
P-value S1 (vs. V1:D0)		0.0938	*0.002*	*0.0039*	*0.0078*	*0.002*	*0.0313*	*0.0313*	0.0625
P-value S2 (vs. V1:D0)		0.0078	*0.0039*	*0.0039*	*0.002*	*0.002*	*0.0313*	*0.0313*	0.0625
P-value (S1 vs. S2)	0.7671	*0.0012*	0.0849	0.0848	0.1003	*<0.0001*	*0.0152*	0.2208	0.3095

Figure 5. Antigen-specific B cell responses induced by MVA-MERS-S vaccination

(A) Representative IgG ELISpot images of antigen-specific and control wells for PBMCs taken before the first (V1:day 0) and after the third vaccination (V3:day 14).

(B) Frequencies of vaccine-induced S1/S2-specific B cells displayed as SFUs/10^6 PBMCs as determined by IgG ELISpot. Data are represented as individual data points (mean of technical duplicates) and median ± IQR. The dotted line indicates the cutoff value (6.6 SFUs/10^6 PBMCs).

(C) p values as determined by Wilcoxon signed rank test (between time points) and Mann Whitney U test (between S1 and S2 responses). Number of samples, median, and p values for each time point are shown in Table S5.

higher numbers of S2- than S1-specific ASBCs (median SFUs: S2 = 23, S1 = 9.5), with 80% of the participants (n = 8/10) showing responses above the cutoff for S2 and 60% (n = 6/10) for S1 at the time point V2:day 14. Prior to late V3 (V3:day 0), S2-specific ASBCs were still present above the cutoff in 60% (n = 6/10) of participants compared with 30% (n = 3/10) for S1-specific B cells. V3 increased the frequency of both S1- and S2-specific ASBCs, resulting in peak responses at day 14 (V3:day 14). All vaccinees (n = 10/10) showed responses above the cutoff for both S subunits at this time point but with higher frequencies of S1- compared with S2-specific ASBCs (median values: S1: 243.8 SFUs, S2: 70.5 SFUs). Comparing peak responses of ASBCs induced by V2 and V3, the number of both S1- and S2-specific ASBCs was significantly higher at V3:day 14 compared with V2:day 14 (Wilcoxon signed rank test: S1: p = 0.002, S2: p = 0.0059), with median fold changes of 24.9 and 2.8 for S1 and S2, respectively. The number of ASBCs decreased following V3:day 14 but persisted above the cutoff throughout the whole study period in 85.7% of all analyzed participants (n = 6/7) for S1 and 28.6% of participants (n = 2/7) for S2 (V3:months 12–24). Notably, the numbers of S1-specific ASBCs detected 12 to 24 months after V3 were higher than those of the peak response after V2, with median values of 22, 17, and 20 SFUs (V3:month 12, V3:month 18, V3:month 24) compared with 9.5 SFUs (V2:day 14). Overall, the number of S2-specific ASBCs was higher following V1 and V2, but S1-specific

ASBCs showed a rapid and strong increase after V3 and remained detectable until the end of the study.

MERS-CoV-S-specific MBCs predominantly belong to the IgG isotype and showed an activated phenotype after late third vaccination

To specifically analyze the induction and phenotype of antigen-specific MBCs, we performed a flow cytometric assay using a combination of fluorescently labeled antigen probes for recognition of MERS-CoV-S-specific B cells (Figures 6A and S2).

The percentage of the total MBC compartment stayed constant over time and was not influenced by vaccinations (Figure S3). However, a detailed analysis of MERS-CoV-S-specific MBCs revealed an increase following MVA-MERS-S vaccination, showing distinct dynamics for each isotype (Figures 6B and S3). For S-specific MBCs of the IgM isotype, no significant changes in the median frequency (MFR) were observed at any time point after V1, V2, or V3 compared with baseline (p values for all time points are shown in Table S6). In comparison, the population of S-specific IgG^+ MBCs showed a first significant increase at V2:day 7 (MFR = 0.15%) with a fold change of 1.9 compared with V1:day 0 (MFR = 0.075%). From this time point on, the population of S-specific IgG^+ MBCs stayed elevated at a significant level until V3:month 18. The frequency of S-specific IgG^+ MBCs ranged from 0.11% to 0.17% between V2:day 7 and V3:day 0 and was strongly enhanced after the late boost, resulting in a fold change of 11.3 for the peak response at V3:day 14 (MFR = 0.89%). Alongside peak responses at V3:day 14, an induction of S-specific MBCs was already observed at V3:day 7 (MFR = 0.36%; fold change = 5.2) and prolonged until V3:day 28 (MFR = 0.885%; fold change = 10.8). Notably, frequencies declined to 0.19% at V3:month 12 but stayed 2.5-fold above baseline until the end of study (V3:month 24). For the IgA isotype, S-specific MBCs revealed no significant changes following V1 and V2, whereas V3 induced significant increases at V3:day 14 (MFR = 0.4%; fold change = 3.8) and V3:day 28 (MFR = 0.17%; fold change = 2.2) compared with V1:day 0 (MFR = 0.09%). The levels declined but stayed above baseline at late time points (V3:month 12 to V3:month 24; fold changes ranging from 1.1 to 1.5).

Since the IgG^+ MBC population showed the strongest induction upon vaccination, we further characterized this subset for activation phenotypes, using the markers CD27 and CD21 (Figure 6C). At V1:day 0, the largest proportion (71.7%) of S-specific IgG^+ MBCs showed a resting phenotype ($CD27^+CD21^+$), whereas activated ($CD27^+CD21^-$), intermediate ($CD27^-CD21^-$), and atypical ($CD27^-CD21^+$) MBCs were less frequent with MFRs of 12.9%, 5.3%, and 9.5%, respectively. Frequencies of activated MBCs were increased at days 7, 14, and 28 following both V2 and V3. Nevertheless, only V3 induced a significant enrichment of the activated MBC compartment with frequencies of 45.2%, 48.9%, and 36.2% at V3:day 7, V3:day 14, and V3:day 28, respectively.

Positive correlations were observed between early and late S2-specific IgG antibody and B cell responses

Correlation analysis was performed between IgG1, IgG3, and ASBC responses at different time points using correlograms specific for S1 and S2 responses. Note that S-specific MBCs were included in both correlograms. Correlations of S1-specific antibody and B cell responses revealed a rather heterogenous picture (Figure 7A) compared with S2-specific responses showing several clusters of positive correlations (Figure 7B). S1-specific responses showed positive correlations between single time points, with the strongest correlations observed for IgG1 (V2:day 14 versus V3:month 18) and IgG3 (V1:day 0 versus V3:month 18) as well as between IgG1 (V1:day 0) versus MBCs (V2:day 14). In comparison, the S2 correlogram revealed positive correlations for all three types of S2-specific responses: IgG1, IgG3, and ASBCs. With regard to IgG1, all time points correlated strongly with each other, while IgG3 responses correlated strongly after V2 and V3 but less so with baseline levels. For IgG1, IgG3, and ASBCs, the responses induced at V3:day 14 correlated with those persisting at V3:month 18. Early responses of both S2-specific ASBCs and S-specific MBCs correlated with S2-specific IgG1 and IgG3 antibody responses at all time points, with the strongest association observed between MBCs and IgG1.

DISCUSSION

The present study aimed at investigating vaccine-induced immunogenicity following V1, V2, and late V3 with the MERS vaccine candidate MVA-MERS-S. Here, we report on the immunogenic potential of a late boost vaccination, providing insight into T and B cell responses and describing for the first time long-term persistence of vaccine-induced MERS-CoV-S1- and -S2-specific antibodies and B cells.

The first two immunizations with MVA-MERS-S (V1 and V2) induced T cell and antibody responses, demonstrating a seroconversion of 100% (n = 11/11) in the high-dose cohort.[16] Besides MVA-MERS-S, two other MERS vaccine candidates have been investigated in phase 1 clinical trials: the DNA-based candidate GLS-5300[18] and the chimpanzee adenoviral vector ChAdOx1 MERS.[17,19] Using a three-dose schedule over 12 weeks and a single shot, the vaccine candidates GLS-5300 and ChAdOx1 MERS induced seroconversion in 94% (n = 61/65) and 100% (high dose, n = 9/9) of the study participants, respectively.[17–19] A recent phase 1b trial testing ChAdOx1 MERS completed the first MERS vaccine trial in Saudi Arabia underscoring an acceptable safety and immunogenicity profile in healthy Middle Eastern adults.[19] Here, seroconversion occurred in 100% (n = 9/9) of participants who received the high dose. All three MERS vaccine candidates were shown to be safe and immunogenic, with antibody levels declining after vaccination but remaining above baseline until 6–12 months post prime immunization.[16–19] T cell responses measured by IFNγ ELISpot were examined in all four studies and showed responses above the cutoff value in the majority of study participants throughout the study period.[16–19]

We extended our original trial by a late V3.[21] While T cell responses following V3 were rather diverse, MERS-CoV-S-specific B cells and antibodies of the IgG isotype as well as neutralizing antibodies were rapidly and homogeneously induced in all vaccinees. In addition, the population of S-specific MBCs not only increased in frequency but was also enriched for activated

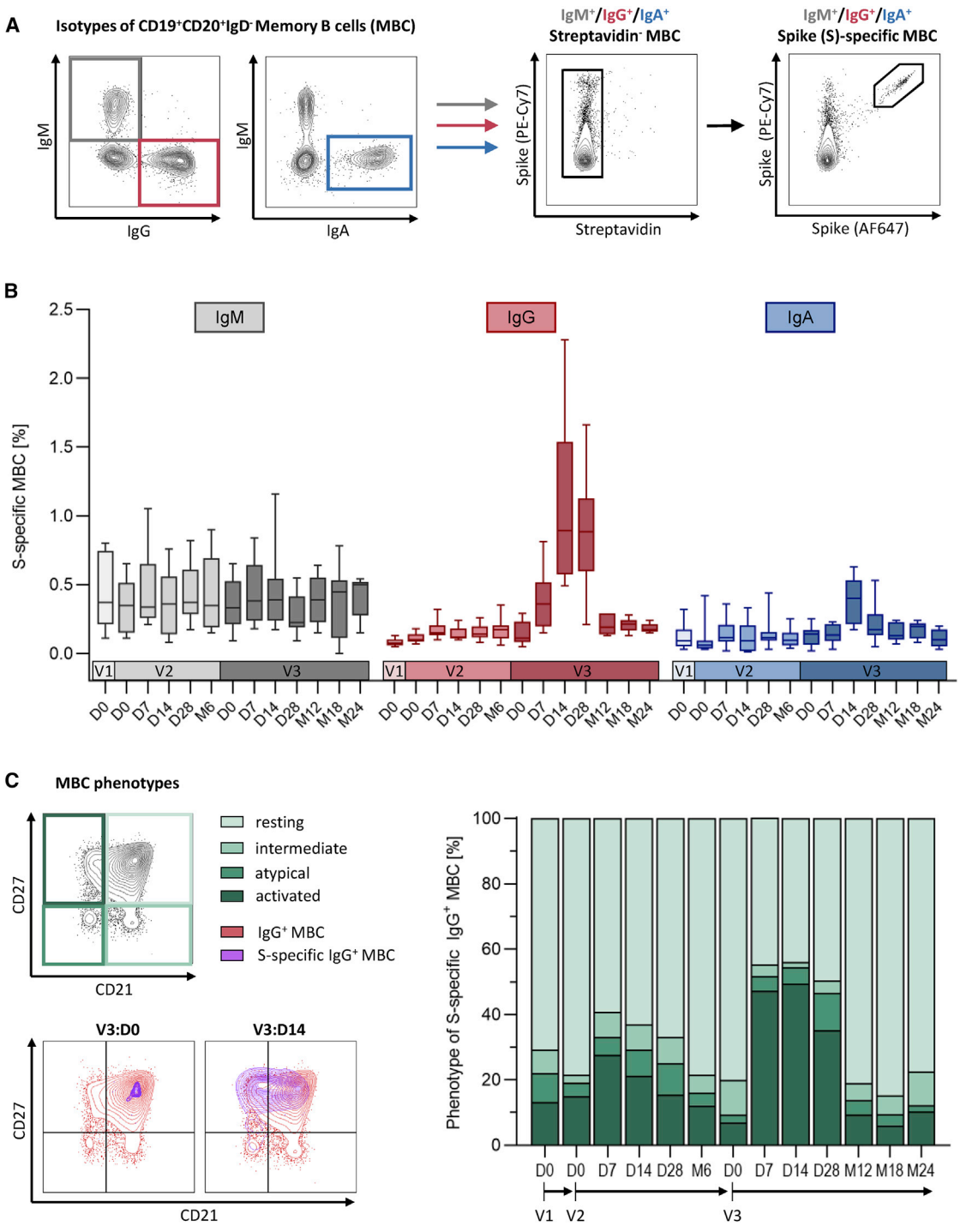

Figure 6. Characterization of vaccine-induced memory B cells

(A) Gating strategy for analysis of isotypes and MERS-CoV-S-specific cells within the memory B cell (MBC) population (representative contour plots belong to time point V3:day 14 from one study participant; gating strategy for identification of MBCs within whole PBMCs shown in Figure S2).

(B) Longitudinal dynamics of antigen-specific MBCs induced by three vaccinations with MVA-MERS-S (V1, V2, and V3). Data are displayed as frequencies of S-specific cells within $IgM^+/IgG^+/IgA^+$ MBCs. Boxplots indicate median ± IQR. Number of samples, median, and p values compared with V1:day 0 are shown in Table S6.

(C) Resting, intermediate, atypical, and activated MBC phenotypes as identified by expression of CD21 and CD27 (top left panel). Representative plots are shown for one study participant at V3:day 0 and V3:day 14 and depict overlaid contour plots of total IgG^+ MBCs and S-specific IgG^+ MBCs (bottom left panel). Longitudinal distributions of phenotypes within the S-specific IgG^+ MBC compartment are shown as mean values of all study participants.

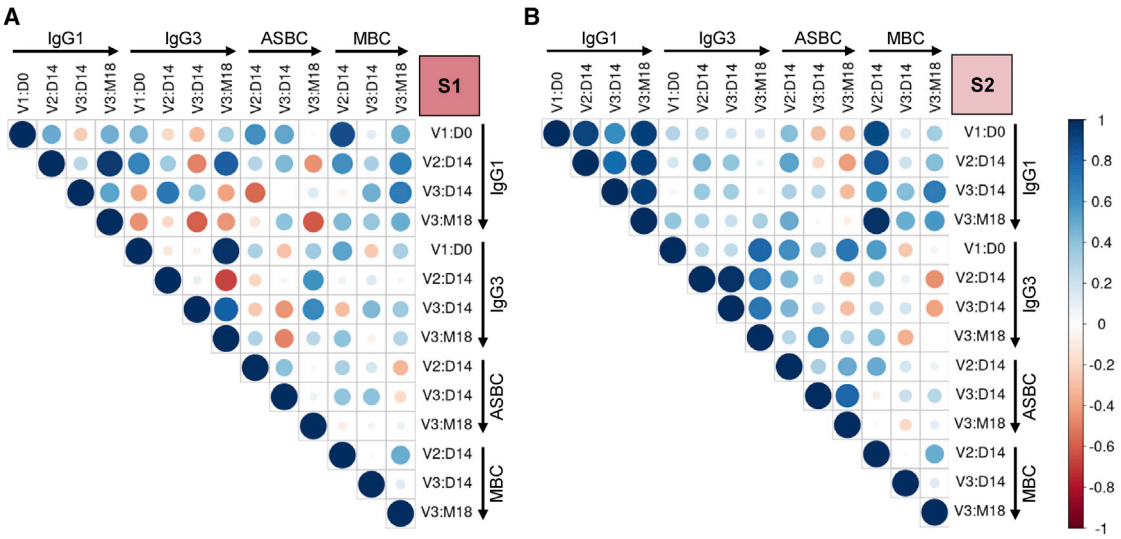

Figure 7. Correlation between vaccine-induced antibody and B cell responses

Correlation analysis of MERS-CoV-S1- (A) and -S2- (B) specific IgG1, IgG3, and ASBC responses at different time points after vaccinations 1, 2, and 3 (V1, V2, and V3). S-specific MBC responses were included into correlograms of both the S1 and S2 antigen. Positive correlations are shown in blue and negative correlations in red, as indicated by the color bar. Sample numbers included into correlation analysis are provided in Table S7.

MBCs ($CD27^{+}CD21^{-}$) resembling plasma cell precursors.[22] This finding, together with antibody responses emerging early after V3 (V3:day 7), provides evidence for the generation of MBCs following V1 and V2, which were then reactivated upon V3. The observed long-lasting S-specific MBCs and ASBCs, but in particular, S1-specific IgG1 and neutralizing antibody responses, in all analyzed participants indicate enhanced induction of not only MBCs but also LLPCs after V3. LLPCs are responsible for maintaining plasma antibody levels, whereas MBCs can rapidly differentiate into antibody-secreting cells upon antigen re-exposure.[23] The strong and robust induction of MERS-CoV-S-specific B cells and antibodies detectable during the entire study period may contribute to preventing infection and shaping the disease course upon MERS-CoV infection. These findings emphasize the relevance of the late boost.

Several factors influence vaccine-induced immune responses, which are often multilayered and can be different for specific vaccine candidates. For example, vector immunity, innate immune responses,[24] and the developmental stage of MBCs[25] can impact the magnitude and quality of boost immune responses. It has been shown that a prolonged interval between prime and boost immunization can be a key element to enhance immunogenicity, as recently demonstrated for the coronavirus 2019 (COVID-19) adenovirus vector vaccine Vaxzevria (AstraZeneca, ChAdOx1 nCoV-19). The vaccine showed stronger immune responses and efficacy when the prime-boost interval was extended from 28 to 84 days.[26,27] The impact of late boosting had been shown previously for the HIV vaccine RV144, where it led to higher IgG titers associated with higher neutralizing capacity,[28] and for an rMVA vaccine against H5N1 (MVA-H5-sfMR), which elicited the highest antibody responses when the boost was administered 1 year after prime immunization.[29] In the latter study, antibody-secreting cells were efficiently induced after the late boost, whereas antigen-specific T cell responses varied considerably within the cohort,[30] a finding comparable to our observations. A recent publication by Munro et al. evaluated the impact of a V3 in a large COVID-19 vaccine trial, in which different prime and boost combinations based on a variety of vaccine platforms were investigated.[31] Different magnitudes of humoral and cellular responses were revealed, depending on the type of vaccine administered for prime and boost immunization. These data highlight the complexity of vaccine-induced immune responses and that specific platforms and intervals might play a critical role for efficient boosting of the humoral, cellular, or both types of immune responses.

In the context of MERS-CoV-S subunits, studies evaluating immune responses to SARS-CoV, SARS-CoV-2, and MERS-CoV have emphasized the importance of responses to the S1 subunit, as they can comprise RBD-specific antibodies capable of blocking interactions with the host cell receptor DPP4. In previous clinical trials testing MERS vaccines, only S1-specific responses were monitored,[16–18] whereas the impact of S2 has been understudied to date. However, a recent murine study emphasized its importance, as two monoclonal antibodies against an S2 epitope showed protection against MERS-CoV in mice.[32] Here, we addressed vaccine-induced MERS-CoV-S2-specific responses in humans for the first time and observed that S2-specific antibodies and ASBCs were already detected following prime immunization in contrast to S1-specific responses, which were earliest detected after the V2. Due to its high conservation among betacoronaviruses, the S2 subunit has been discussed as a target for cross-reactive antibodies resulting from previous infections with different species of human CoVs (HCoVs). Particularly in the case of SARS-CoV-2 infection or vaccination, it is currently discussed that the conserved S2 subunit may trigger S2-specific responses early after antigen

encounter, and cross-reactivity was especially reported toward HCoV-OC43.[33] The isolated MERS-CoV-S2-specific monoclonal antibodies from the above-mentioned mouse study were also cross-reactive to HCoV-OC43.[32] Our study participants were screened for pre-existing antibodies against the HCoVs HCoV-OC43, HCoV-229E, HCoV-HKU1, and HCoV-NL63 prior to the V1 with MVA-MERS-S.[21] Pre-existing antibodies against HCoV-OC43 correlated with S2-specific ASBC responses after V1 and V2 and, to a lesser extent, with S1-specific ASBCs induced by V2 (Figure S4), whereas no correlations with other HCoVs were observed. Comparing antibodies and B cells induced by MVA-MERS-S, positive correlations were specifically observed between early and late time points of S2-specific responses, further underlining a potentially higher dependence of S2-specific responses on pre-existing levels of immunity compared with S1-specific responses, which may result in stronger S2-specific responses after V1. However, the role of early, cross-reactive S2-specific responses in preventing infection and modulating disease progression warrants further investigation.

The comprehensive analysis of antibody isotypes and subclasses may contribute to a better understanding of vaccine-induced protection, as they differ in their capacity to induce non-neutralizing effector functions whose relevance has been shown for a variety of infections and vaccinations.[34–38] While neutralizing antibodies are critical for the prevention of virus entry into the host cell, non-neutralizing antibody functions are increasingly recognized as important mediators of virus control. The antibodies induced by MVA-MERS-S predominantly belong to the IgG1 and IgG3 subclasses, which are known to bind human Fc receptors with high affinity and to be potent activators of effector cells.[39] Notably, a similar pattern of IgG1 and IgG3 induction has also been observed following immunization with the adenoviral vector vaccine Vaxzevria.[40] IgG3 is a pro-inflammatory antibody subclass that has been associated with enhanced protection against viruses, e.g., in a trial of the RV144 HIV vaccine,[35,41,42] and the here-observed robust IgG3 responses against the conserved S2 subunit may represent a critical element in the context of non-neutralizing functions. Future studies should include investigations on the functionality of antibodies by using systems serology approaches to better understand the full impact of MVA-MERS-S prime and boost immunizations on the quality of vaccine-induced antibodies.

Important aspects that need to be addressed in future trials are the exact contribution of dosing intervals, distinct boosting strategies, and heterologous prime-boost regimens to immunogenicity of MERS vaccines. While data on correlates of protection (CoPs) are still very limited for MERS-CoV, we can gain first insights into CoPs from SARS-CoV-2 infection. Here, neutralizing antibodies are suggested to be critical for protection against infection,[43,44] while protection against severe disease is assumed to be at least partially driven by T cell responses.[45] The importance of neutralizing antibodies against MERS has been recently emphasized by Kim and colleagues. Using passive transfer of neutralizing antibodies from MERS survivors to mice, they observed significantly reduced viral loads and increased survival rates, suggesting a protective effect of neutralizing antibodies.[46] Whether MBCs can be used as a CoP, analogous to the hepatitis B context,[47] remains unknown to date but was recently suggested in a publication on SARS-CoV-2 Delta-variant breakthrough infections. In this study, a lower frequency of virus-specific MBCs was detected in patients with breakthrough infection, providing first evidence that MBCs might be used as a CoP.[48] Our data on MVA-MERS-S highlight its potential to induce robust and long-lasting antibody and B cell responses and represent a promising basis for future studies. Whether these factors mediate protection against infection and/or disease severity and death is not addressable with the data presented here.

The here-observed kinetics of antibody persistence show similarities to those observed in MERS survivors, as reported by three studies monitoring their adaptive immune responses.[46,49,50] Sustained antibody titers were observed for 3 to 5 years, especially in individuals that suffered from severe MERS. In comparison, survivors with mild or asymptomatic disease revealed a more variable response with lower titers. In the study by Cheon et al., binding antibodies were detected for up to 4 years after infection and significantly dropped in the 5th year, whereas the neutralizing antibody titers against MERS-CoV decreased more rapidly and were significantly reduced at 4 years after infection.[49]

Taken together, our study demonstrates that MVA-MERS-S induced robust MERS-CoV-S-specific B cells and antibodies in a homologous vaccination regimen, whereas T cell responses displayed a more heterogenous pattern within the cohort. Magnitude and persistence of both MERS-CoV-S-specific B cells and antibodies were strongly enhanced by the late third vaccination. Antibody levels and neutralization capacity seemed to be stabilized after the late boost. This work adds evidence to a growing number of reports, demonstrating that late boosting could be an important tool for improving vaccine-induced immunogenicity against CoVs. A vaccination schedule using a long interval could be of particular interest in a pre-pandemic situation to establish long-lasting S-specific antibodies in the target population, e.g., healthcare and camel workers, while a short interval or even single-shot immunizations are crucial in outbreak situations. This pilot study using MVA-MERS-S forms the basis for future studies and may also provide translatable insights into long-term immune responses to COVID-19 vaccines and the application of late-boosting strategies.

Limitations of the study

In our study, we longitudinally evaluate adaptive immune responses after immunization with the MVA-MERS-S vaccine candidate. One limitation of our study is the small size of the study cohort, which consists of ten study participants who received three vaccinations and were subsequently monitored until 28 days after the last vaccination. During the long-term follow up (12–24 months after the third vaccination), seven of the ten study participants continued with further visits. A second limitation of our study is the usage of PBMCs to study MBC responses. While peripheral blood provides first insights into the induction of antigen-specific cells following vaccination, analyses of lymphoid tissue and bone marrow would be additionally important to investigate vaccine-induced B and T cell populations.

Furthermore, we did not evaluate the induction of vaccine-induced mucosal responses as we did not sample biopsies from the pharyngeal mucosa. In addition, the impact of the observed immune responses on protection against MERS-CoV infection and disease remains an open question that cannot be addressed with our study.

CONSORTIA

The members of the MVA-MERS-S Study group are Etienne Bartels, Swantje Gundlach, Thomas Hesterkamp, Verena Krähling, Susan Lassen, My Linh Ly, Joseph H. Pötsch, Stefan Schmiedel, Asisa Volz, and Madeleine E. Zinser.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Vaccine construct
  - Study design and participants
  - Blood sampling
- METHOD DETAILS
  - ELISA
  - PRNT50
  - IFNγ ELISpot assay
  - Bead-based multiplex ELISA
  - IgG ELISpot assay
  - Flowcytometry
- QUANTIFICATION AND STATISTICAL ANALYSIS
- ADDITIONAL RESOURCES

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xcrm.2022.100685.

ACKNOWLEDGMENTS

We thank all volunteers for their participation in this first-in-human phase 1 vaccine trial and their commitment and dedication to research against emerging CoVs. We would also like to express our sincere gratitude to all trial center members for their extraordinary work (Clinical Trial Center North GmbH & Co. KG, Hamburg), especially Saskia Borregaard, Alen Jambrecina, and Laura Kaltenberg. We also thank Keith Chappell (University of Queensland) for providing MERS-CoV-S clamp antigen. This work was funded by the DFG grant AD171/3-1 and the DZIF infrastructure "Clinical management and epidemiology of emerging infections" [01.702] and FKZ8009801908, FKZ8009701702, and FKZ80095CLANF.

AUTHOR CONTRIBUTIONS

Conceptualization, M.M.A., S.B., G.S., A.F., T.K., and C.D.; methodology, L.M.W., C.D., M.P.R., and B.L.H.; investigation: L.M.W., M.F., M.P.R., A.Z.M., and the MVA-MERS-S Study Group; formal analysis and visualization, L.M.W. and M.S.; writing – original draft, L.M.W. and C.D.; writing – review & editing, L.M.W., C.D., M.M.A., A.F., T.K., G.S., and B.L.H.; supervision, C.D. and M.M.A.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: October 8, 2020
Revised: February 25, 2022
Accepted: June 16, 2022
Published: July 19, 2022

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse Anti-Human IgM-PE	SouthernBiotech	Cat#9020-09; RRID:AB_2796586
Mouse Anti-Human IgA1-PE	SouthernBiotech	Cat#9130-09; RRID:AB_2796656
Mouse Anti-Human IgA2-PE	SouthernBiotech	Cat#9140-09; RRID:AB_2796664
Mouse Anti-Human IgG1 Hinge-PE	SouthernBiotech	Cat#9052-09; RRID:AB_2796621
Mouse Anti-Human IgG2 Fc-PE	SouthernBiotech	Cat#9070-09; RRID:AB_2796639
Mouse Anti-Human IgG3 Hinge-PE	SouthernBiotech	Cat#9210-09; RRID:AB_2796701
Mouse Anti-Human IgG4 Fc-PE	SouthernBiotech	Cat#9200-09; RRID:AB_2796693
PerCP anti-human CD3 Antibody	Biolegend	Cat#344813; RRID:AB_10641841
PerCP anti-human CD14 Antibody	Biolegend	Cat#301847; RRID:AB_2564058
Alexa Fluor® 700 anti-human CD56 (NCAM) Antibody	Biolegend	Cat#318316; RRID:AB_604104
Brilliant Violet 650™ anti-human CD20 Antibody	Biolegend	Cat#302335; RRID:AB_11218609
Brilliant Violet 785™ anti-human IgD Antibody	Biolegend	Cat#348241; RRID:AB_2629808
Brilliant Violet 570™ anti-human IgM Antibody	Biolegend	Cat#314517; RRID:AB_10913816
BD OptiBuild™ BUV805 Mouse Anti-Human IgG	BD	Cat#742041; RRID:AB_2871333
BUV563 Mouse Anti-Human CD19	BD	Cat#612917; RRID:AB_2870202
BD Horizon™ BUV737 Mouse Anti-Human CD21	BD	Cat#612788; RRID:AB_2870115
BD Horizon™ BB515 Mouse Anti-Human CD27	BD	Cat#564643; RRID:AB_2744354
IgA Antibody anti-human, APC-Vio 770	Miltenyi Biotec	Cat#130-113-999; RRID:AB_2733153
Rabbit anti-MERS-CoV Nucleoprotein antibody	Genetex	Cat#GTX134868; RRID:AB_2887364
Goat anti-Rabbit IgG antibody, Alexa Fluor 488	Invitrogen	Cat#A32731; RRID:AB_2633280
Bacterial and virus strains		
MERS-CoV, EMC/2012 isolate		GenBank accession no. NC_019843.3
Biological samples		
Cryopreserved PBMC from MVA-MERS-S vaccinees	University Clinical Center Hamburg-Eppendorf, Germany	N/A
Cryopreserved plasma from MVA-MERS-S vaccinees	University Clinical Center Hamburg-Eppendorf, Germany	N/A
Cryopreserved serum from MVA-MERS-S vaccinees	University Clinical Center Hamburg-Eppendorf, Germany	N/A
Chemicals, peptides, and recombinant proteins		
Recombinant clamp MERS-CoV-spike protein	Keith Chappell, The School of Chemistry and Molecular Biosciences, University of Queensland, Brisbane, QLD, Australia	N/A
MERS peptide pool 1-5	JPT Peptide Technologies	Customized; Sequences indicated in Table S8
CEF Pool (extended)	JPT Peptide Technologies	Cat#PM-CEF-E−3
Phytohemagglutinin (PHA) (1 mg/mL)	Sigma	Cat#L8902-5MG
MERS-CoV Spike Protein (S1 Subunit, aa 1-725, His Tag)	Sino Biological	Cat#40069-V08B1
MERS-CoV Spike Protein (S2 Subunit, aa 726-1296, His Tag)	Sino Biological	Cat#40070-V08B
MERS-CoV Spike S1+S2 Protein (ECD, aa 1-1297, His Tag), Biotinylated	Sino Biological	Cat#40069-V08B-B

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
LIVE/DEAD™ Fixable Blue Dead Cell Stain Kit	Thermo Fisher	Cat#L23105
Streptavidin, (PE-Cy5.5)	Thermo Fisher	Cat#SA1018
AF647 Streptavidin	Biolegend	Cat#405237
PE-Cy7 Streptavidin	Biolegend	Cat#405206
BIO200 - BIO-200 biotin solution	Avidity	Cat#BIO200
Critical commercial assays		
CTL Human IFN-g Single colour 384- well Enzymatic ELISpot Kit	ImmunoSpot	Cat#hIFNg-3M/5
Human IgG ELISpot BASIC kit (ALP)	Mabtech	Cat#3850-2A
Software and algorithms		
FlowJo v10.8.0	FlowJo, LLC	https://www.flowjo.com/
GraphPad Prism v8.0.1	GraphPad	https://www.graphpad.com
R v4.0.2	R Foundation	https://www.r-project.org/foundation/
RStudio	RStudio	https://www.rstudio.com/
Elispot Reader v7.0 (build 16577)	AID GmbH	https://www.elispot.com/
Bio-Plex Manager™ Software v6.2 (build 175)	Bio-Rad Laboratories, Inc.	https://www.bio-rad.com/
SpectroFlo® v3.0.1	Cytek Biosciences	https://cytekbio.com/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Christine Dahlke (c.dahlke@uke.de).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Vaccine construct

MVA-MERS-S is based on a rMVA vector encoding for the full-length MERS-CoV-S-glycoprotein, based on the sequence of EMC/2012 (GenBank accession no. JX869059). MVA-MERS-S expresses the full-length S protein of MERS-CoV with a molecular mass closely corresponding to the mass predicted from the S gene nucleotide sequence and evidence for an S1 and S2 cleavage of full-length S.[20] The cDNA sequence was not codon-optimized in the classical sense. It was obtained by gene synthesis and modified by introducing silent codon alterations to inactivate three signal sequences (TTTTTNT) for termination of vaccinia virus-specific early transcription. This modification allows for optimized gene expression when using early-late promotors for MVA-specific transcription of recombinant genes. The vaccine was manufactured by IDT Biologika GmbH (Dessau, Germany) in primary chicken embryo fibroblasts (CEF).

Study design and participants

NCT03615911 was a phase 1 clinical trial to address safety and immunogenicity of the vaccine candidate MVA-MERS-S in healthy adults. The study was conducted in Hamburg (Germany) at the University Medical Center Hamburg-Eppendorf (UKE). Eligible adults were males or females aged between 18 and 55 without previous MVA-immunization. Study participants were divided into two dose groups that received either 1×10^7 plaque-forming units (PFU, low dose) or 1×10^8 PFU (high dose) on days 0 and 28 [16]. A subgroup of participants (3 male, 7 female) from the low dose (n = 3) and the high dose (n = 7) groups received a late booster immunization of 1×10^8 PFU MVA-MERS-S 12 ± 4 months after prime immunization[21] and represents the core study population of this manuscript. The study design of the clinical trial was reviewed and approved by the Competent National Authority (Paul-Ehrlich-Institut, PEI,

Langen, Germany) and the Ethics Committee of the Hamburg Medical Association and is registered at ClinicalTrials.gov (NCT03615911). The observational study was approved by the Ethics Committee of the Hamburg Medical Association and is registered under Protocol No. PV6079. All studies were performed in accordance with the Declaration of Helsinki in its version of Fortaleza 2013. All participants provided written informed consent prior to enrollment in the studies.

Blood sampling

Serum and EDTA blood were sampled from all study participants (n = 10) before vaccination 1 (V1:D0), at days 0, 7, 14, 28 and 152 (= 6 months) after vaccination 2 (V2:D0, V2:D7, V2:D14, V2:D28, V2:M6) and at days 0, 7, 14 and 28 after vaccination 3 (V3:D0, V3:D7, V3:D14, V3:D28). Additionally, blood was sampled at 12, 18 and 24 months after V3 from some of the participants (V3:M12, V3:M18, V3:M24). For the exact days of blood sampling and the number of participants sampled late after V3 see Table S1. PBMC were isolated from EDTA blood via Ficoll separation and cryopreserved in liquid nitrogen. Plasma and serum samples were stored at −80°C.

METHOD DETAILS

ELISA

Total anti-MERS-CoV-S IgG was measured using a standardized in-house indirect ELISA. High binding 96-well microplates were coated at 4°C overnight with 100 μL of full-length recombinant clamp MERS-CoV-S protein (1 μg/mL, supplied by Keith Chappell, University of Queensland). Plates were blocked using 100 μL blotto in TBS (ThermoFisher Scientific) per well for 60 min at 37°C. Plates were washed three times after each incubation step, using PBS with 0.05% (v/v) Tween 20. Sera were diluted 1:100 in blocking buffer and 100 μL of diluted serum was incubated on the coated plates at 37°C for 60 min. Antibody staining was performed using 100 μL of horseradish peroxidase (HRP)-conjugated rabbit-anti-human IgG secondary antibody (1.3 mg/mL, Dako) in 1:6000 dilution in PBS for 60 min at 37 °C. Enzymatic reaction was initiated by adding 100 μL 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate for 5 min at room temperature. The reaction was stopped by adding 100 μL of 0.25 M sulfuric acid. Photometry was performed using a microplate reader (Tecan Infinite F200) at a measurement wavelength of 450 nm with a reference wavelength of 620 nm. Results were reported as optical density (OD) values of the measurement wavelength, subtracted by the reference wavelength. The cut-off OD value for positivity was set at > 0.1, above the geometric mean OD value of negative control sera +3 standard deviations (0.094).

PRNT50

Serum samples were heat inactivated at 56°C for 30 min 50 μL of serum were serially diluted by 2-fold in Opti-MEM I (1×) + GlutaMAX (Gibco), mixed 1:1 with 400 PFU of MERS-CoV (EMC/2012 isolate; GenBank accession no. NC_019843.3) and incubated at 37°C for 1 h. The mixture was then transferred to Calu-3 cell monolayers maintained in Opti-MEM I (1×) + GlutaMAX (Gibco) supplemented with 10% Fetal Bovine Serum (FBS), penicillin (100 IU/mL), and streptomycin (100 IU/mL). Cells were incubated at 37°C and 5% CO_2 for 8 h and subsequently fixed and permeabilized with formalin and 70% ethanol, respectively. Cells were washed with phosphate buffered saline (PBS) and blocked in 0.6% bovine serum albumin (BSA). Stainings were performed using rabbit anti-MERS-CoV nucleocapsid antibody (Genetex, 1:2000 in 0.1% BSA in PBS) followed by goat anti-rabbit Alexa Fluor 488 antibody (Invitrogen, 1:4000 in 0.1% BSA in PBS). Stained plates were scanned on the Amersham Typhoon Biomolecular Imager (GE Healthcare) and infection was quantified using ImageQuantTL 8.2 image analysis software (GE Healthcare). The PRNT50 titer was quantified using non-linear regression analysis in Graphpad Prism 9.

IFNγ ELISpot assay

IFNγ secretion by T cells was analyzed using a CTL Human IFN-γ Single color 384-well Enzyme-linked Immuno Spot Assay (ELISpot, ImmunoSpot®). Cryopreserved PBMC were thawed, rested overnight and plated at 5 × 10^5 PBMCs per well in serum-free medium (CTL medium, ImmunoSpot®). PBMC were stimulated with OLP pools M1-M5, spanning the entire amino acid sequence of MERS-CoV-S protein (final concentration: 1 μg/mL; for peptide sequences see Table S8) for 16 h at 37°C and 9% CO_2. While incubation with phytohemagglutinin (PHA) and a CMV/EBV/Influenza (CEF) peptide pool (JPT Peptide Technologies) served as positive controls, negative controls were incubated with CTL medium plus Dimethyl sulfoxide (DMSO) at the same concentration used for the reconstitution of the MERS-CoV-S peptide pools. Spot detection, ELISpot image acquisition and analyses were performed using the AID EliSpot Reader System (AID GmbH). Spot forming units (SFU) were calculated using the geometric mean of triplicates. DMSO controls were used to normalize the data. A positive response was defined using two criteria: first, a response >50 SFU per 1 M PBMCs and secondly, a four-fold value above baseline (V1:D0).

Bead-based multiplex ELISA

A bead-based multiplex ELISA was used to determine the proportion of isotypes and subclasses within MERS-CoV-S-specific antibodies. Two regions of carboxylated microspheres (Luminex) were covalently coupled with either the S1 or S2 subunit of MERS-CoV-S (SinoBiological) as described previously.[51] Microspheres of both regions were diluted to 50,000/mL in PBS containing 0.1% BSA and added to a black, clear-bottom 96 well microplate at 50 μL (Greiner Bio-One). 50 μL of plasma sample diluted 1:50 in 0.1% BSA/PBS were added and incubated overnight at 4°C and 850 rpm on an orbital shaker. The microspheres were washed with 0.1% BSA/PBS containing 0.5% Triton X-(3×) and with 0.1% BSA/PBS (1×). PE-conjugated detection antibodies specific for

human IgM, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2 (Southern Biotech) were added to individual wells for detection of microsphere-bound plasma antibodies. Plates were incubated for 2 h at room temperature and 850 rpm, washed and read out on a Bio-Plex 200 System. The cut-off value was defined as 2-fold above baseline. All measurements were performed in duplicates and the mean of both wells was used for further analysis. Microspheres incubated with detection antibodies in absence of plasma sample (blank wells) were measured as a control for unspecific background signal and used for calculation of the lower limit of detection (LLD) of each analyte: LLD = mean (normalized blank) + 3 × standard deviation (normalized blank).

IgG ELISpot assay

MERS-CoV-S-specific B cells were analyzed using an antigen-specific IgG ELISpot. Cryopreserved PBMC were thawed, resuspended to 2×10^6 cells/mL and stimulated in R10 containing 1% Hepes (Thermo Fisher Scientific), 0.5 μg/mL resiquimod (R848, Mabtech) and 5 ng/mL interleukin-2 (IL-2, Mabtech) for 75 h at 37°C and 5% CO_2. PVDF-Multi-Screen-IP plates (Millipore) were treated with 15 μL/well 35% ethanol and washed with sterile water. Plates were coated overnight at 4°C with 100 μL/well of either PBS containing anti-IgG capture antibody (15 μg/mL, Mabtech), MERS-CoV-S protein S1 or S2 subunit (10 μg/mL, SinoBiological), or PBS only. Plates were washed and after 30 min blocking with R10 containing 1% Hepes, pre-stimulated PBMC were added to two replicate wells of each coating condition and incubated for 16 h at 37°C and 5% CO_2. For detection of spots, biotinylated anti-IgG detection antibody, streptavidin-ALP and BCIP/NBT-plus substrate solution (Mabtech) were used according to the manufacturer's protocol. Plates were analyzed using an AID EliSpot Reader System (AID GmbH). The cut-off value was set at 6.6 SFU, calculated as the geometric mean of blank wells +2 standard deviations.

Flowcytometry

Multiparametric flow cytometry was used to analyze the isotype and activation phenotype of MERS-CoV-S-specific memory B cells. Antigen probes for detection of antigen-specific B cells were prepared in advance by multimerization of biotinylated MERS-CoV-S antigen (SinoBiological) with fluorescently labeled streptavidin (SA). To be able to exclude B cells binding to SA and the respective fluorophore, SA-PE/Cy5.5 (Thermo Fisher) was added as a decoy probe and MERS-CoV-S was separately multimerized with SA labeled with two different fluorophores. For multimerization, MERS-CoV-S protein was mixed with SA-PE/Cy7 and SA-AF647 (Biolegend), respectively, at a molar ratio of 4:1 (mass ratio 11:1) and incubated for 60 min at 4°C. Cryopreserved PBMC were thawed and distributed in a 96 well V-bottom plate at 5–10 × 10^6 PBMC per sample. Cells were first stained with FcR blocking reagent (Miltenyi Biotec, 1:20) and LIVE/DEAD Fixable Blue (Thermo Fisher, 1:1000) in 100 μL of FACS Buffer (PBS containing 2% FBS and 2 mM EDTA) for 15 min at 4°C. Cells were then washed and stained with 50 μL of Brilliant Stain Buffer (BD) containing 5 μM free d-biotin (Avidity), 165 ng spike-PE/Cy7, 165 ng spike-AF647 and 20 ng SA-PE/Cy5.5 decoy probe for 60 min at 4°C. Cells were washed again and subsequently stained with 100 μL of FACS Buffer/Brilliant Stain Buffer (1:1) mixed with 2.5 μL of antibodies against human CD3, CD14, CD56, CD19, CD20, CD21, CD27, IgD, IgM, IgG and IgA (see key resource table) for 30 min at 4°C. After surface staining, cells were washed and fixed with 4% PFA for 15 min at room temperature. Cells were washed again, resuspended in 100 μL of FACS Buffer and acquired using a Cytek® Aurora (Cytek Biosciences).

QUANTIFICATION AND STATISTICAL ANALYSIS

Flowcytometry data was analyzed using FlowJo software v.10. Statistical analysis was performed using GraphPad Prism (v8.0.1). Statistical testing was conducted by using two-tailed Wilcoxon signed-rank and Mann-Whitney U tests for paired and unpaired samples, respectively, and the level of significance was set to 0.05. The number of study participants (n), median and p values for all experiments are shown in Tables S2–S7. The number of study participants differs between experiments at some time points because samples were not always sufficient to perform all experiments. Correlations between ELISA and PRNT50 data (Figure 2C) as well as between B cells and HCoV antibodies (Figure S4) were calculated with GraphPad Prism using non-parametric Spearman's correlation. Correlations within MERS-CoV datasets and the plots based on them were rendered with R (v4.0.2) and R package corrplot (v0.84) (using Visualization of a Correlation Matrix (v0.84), available from https://github.com/taiyun/corrplot).

ADDITIONAL RESOURCES

ClinicalTrials.gov Identifier: NCT03615911. https://clinicaltrials.gov/ct2/show/NCT03615911.