Marburg virus survivor immune responses are Th1 skewed with limited neutralizing antibody responses

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Until recently, immune responses in filovirus survivors remained poorly understood. Early studies revealed IgM and IgG responses to infection with various filoviruses, but recent outbreaks have greatly expanded our understanding of filovirus immune responses. Immune responses in survivors of Ebola virus (EBOV) and Sudan virus (SUDV) infections have provided the most insight, with T cell responses as well as detailed antibody responses having been characterized. Immune responses to Marburg virus (MARV), however, remain almost entirely uncharacterized. We report that immune responses in MARV survivors share characteristics with EBOV and SUDV infections but have some distinct differences. MARV survivors developed multivariate CD4+ T cell responses but limited CD8+ T cell responses, more in keeping with SUDV survivors than EBOV survivors. In stark contrast to SUDV survivors, rare neutralizing antibody responses in MARV survivors diminished rapidly after the outbreak. These results warrant serious consideration for any vaccine or therapeutic that seeks to be broadly protective, as different filoviruses may require different immune responses to achieve immunity.

INTRODUCTION

The genera *Marburgvirus* and *Ebolavirus* comprise the family Filoviridae, which contains the etiological agents that cause Marburg virus (MARV) disease and Ebola virus (EBOV) disease, respectively. EBOV recently caused an outbreak of unprecedented scale, spanning 2 yr and infecting >28,000 individuals. Previously, the largest filovirus outbreaks had numbered in the tens to hundreds. Whereas EBOV is responsible for the greatest number of human filovirus infections, MARV, Sudan virus (SUDV), and Bundibugyo virus (BDBV) have all caused outbreaks in humans that numbered >100 cases, twice in the cases of MARV and SUDV. EBOV may garner much of the attention, but it is only one of several filoviruses that could potentially kindle outbreaks of massive scale. To date, there have been 11 incidences of MARV transmission to the human population that spawned outbreaks of varying size. Whereas many outbreaks have been of limited scale, afflicting fewer than five individuals per instance, two outbreaks alone account for 406 cases of MARV infections that had fatality rates of 83 and 90% (Bausch et al., 2006; Towner et al., 2006). Furthermore, a precedent already exists for the importation of MARV to other countries from travelers that visited MARV hot spots (Centers for Disease Control and Prevention (CDC), 2009; van Paassen et al., 2012), which was a concern during the 2013–2015 EBOV outbreak in western Africa. Simple luck or coincidence has thus far prevented these MARV outbreaks from growing into larger problems.

In 2012 alone, four distinct filovirus outbreaks occurred in Africa. Three outbreaks occurred in Uganda (two of SUDV and one of MARV), with the fourth occurring in the Democratic Republic of Congo (BDBV; Albariño et al., 2013). Sequencing of viral genomes isolated from two fatal cases of MARV in 2012 indicated a high similarity with the original MARV isolate (Amman et al., 2012; Albariño et al., 2013). MARV case fatality rates (CFRs) vary from 23 to 90% in cases where more than one individual was infected. The CFR for the MARV outbreak in 2012 was 27% ($n = 15$ confirmed cases), in keeping with the outbreak that was caused by the MARV isolate bearing the greatest similarity to the 2012 isolates (original MARV isolate in 1967; CFR of 23%; Bukreyev et al., 1995; Slenczka and Klenk, 2007; Amman et al., 2012; Albariño et al., 2013).

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Profiles of immunity developed in filovirus survivors have begun to shed light on immune responses that had been understudied. Antibody responses to EBOV had previously been relatively well characterized, with IgM responses being succeeded by IgG in survivors, whereas nonsurvivors fail to develop IgG responses (Baize et al., 1999; Ksiazek et al., 1999) Additionally, neutralizing antibody responses have been described in cases of EBOV, SUDV, BDBV, and a single case of MARV infection (Maruyama et al., 1999; Sobarzo et al., 2012; Flyak et al., 2015, 2016; Bornholdt et al., 2016). A recent study has directly shown that EBOV survivors treated in the U.S., either after exposure in western Africa or after nosocomial infection in the U.S., developed IFN-γ– and TNF-positive CD4$^+$ and CD8$^+$ T cell responses to various EBOV proteins (McElroy et al., 2015). This was the first time antigen-specific responses in T cells had been directly demonstrated in human survivors of filovirus infection. A subset of EBOV-infected individuals also had elevated levels of serum IL-2, IFN-γ, and IL-4 that were measured longitudinally during their treatment in the U.S. (McElroy et al., 2016). IL-2, IFN-γ, and TNF were also found to be present in serum samples from survivors and nonsurvivors of an EBOV outbreak in the Democratic Republic of Congo (Villinger et al., 1999), whereas TNF was found to be decreased and IFN-γ showed no statistical change in serum from BDBV-infected individuals (Gupta et al., 2012). Levels of these cytokines were not reported in a broad cytokine analysis of serum from the 2000–2001 SUDV outbreak (McElroy et al., 2014), but previous studies of serum from that outbreak showed mixed results for TNF and IFN-γ by ELISA and quantitative PCR analyses (Sanchez et al., 2004; Hutchinson and Rollin, 2007). A later study revealed the presence of CD4$^+$ T cell responses in the same SUDV survivor pool, measured >10 yr after the outbreak (Sobarzo et al., 2016). Collectively, these studies have provided a detailed description of adaptive immune responses in survivors of EBOV, SUDV, and BDBV infection.

It is unknown whether immune responses from MARV survivors will present similar profiles to those described for other filoviruses, or if there will be critical differences. Beyond IgG or IgM ELISAs performed for diagnostic purposes, virtually the entirety of the literature available regarding filovirus immune responses in humans comes from studies of survivors of EBOV, SUDV, and BDBV infection. In this study, we provide the first comprehensive immune profiling of responses in human MARV survivors. Identification of a consensus immune response elicited by filovirus infection would be highly valuable for the evaluation of vaccines and therapeutics against MARV disease and those that would seek to be broadly protective.

RESULTS AND DISCUSSION
T cell cytokine responses
Heparinized blood samples from six survivors of the 2012 MARV outbreak in Uganda, as well as local individuals who had not been infected, were obtained ∼9 mo after the res-

olution of the outbreak. Our use of whole blood cultures to measure filovirus survivor immune responses has been described previously (Sobarzo et al., 2016). Whereas many valuable phenotypic analyses on bulk T cell populations have been conducted on acute-phase samples from EBOV-infected individuals (McElroy et al., 2015; Agrati et al., 2016; Ruibal et al., 2016), we deemed a functional analysis of virus-specific immune responses to be more informative, as the samples in our studies were far removed from the acute infection. Unfortunately, we were unable to procure acute-phase samples from the 2012 MARV outbreak; however, such phenotypic studies would be invaluable should samples be available in future outbreaks.

A representative set of flow cytometry plots for CD4$^+$ and CD8$^+$ T cell responses from a MARV survivor is shown in Fig. 1 A. Plots are gated sequentially on lymphocytes, singlets, live cells, and CD3$^+$, CD4$^+$, or CD8$^+$ events (Sobarzo et al., 2016). Resting, SUDV glycoprotein (GP), MARV GP, and irradiated SUDV cultures elicited minimal to no cytokine secretion from either CD4$^+$ or CD8$^+$ T cells. Irradiated MARV stimulation elicited cytokine expression in survivor CD4$^+$ T cells (Fig. 1 A, top), comprising IFN-γ and IL-2 double- and single–positive responses. Survivor CD8$^+$ T cells (Fig. 1 A, bottom) displayed a more limited response, consisting almost exclusively of IFN-γ production. Cells from uninfected control individuals showed no reactivity to irradiated MARV or SUDV or the respective recombinant GP (not depicted).

Individual survivors' CD4$^+$ T cell responses are depicted in Fig. 1 B. IFN-γ and IL-2 single- or double-positive responses to irradiated MARV stimulation are shown for each survivor. Because only the irradiated MARV stimulation resulted in cytokine production, we focused further on that response to determine its composition. Resting values were subtracted to account for nonspecific responses. All but one survivor (S1) had IFN-γ single–positive responses, whereas all survivors had IFN-γ, IL-2 double-positive, and IL-2 single–positive responses. The magnitude of each individual's response varied greatly, with some survivors having small but detectable responses (S1 and S6), whereas others were very robust (S2 and S5; Fig. 1 B). Levels of CD8$^+$ T cell responses were low and did not permit a similar analysis (Fig. 1 A and not depicted).

Secreted cytokine analysis
To complement the flow cytometry analysis, we performed a multiplex ELISA assay with the culture supernatants to analyze a broader range of secreted cytokines. We focused on five cytokines that are germane to adaptive immune responses: IL-2, IFN-γ, TNF, IL-4, and IL-5. Mean resting and irradiated SUDV-stimulated expression levels of each cytokine were low for uninfected control and survivor samples. MARV stimulation elicited IL-2, IFN-γ, and TNF expression that was significantly higher than resting and SUDV-stimulated cultures (P < 0.05; Fig. 2). IL-4 was not measured in any of the cultures, and only a slight, insignificant increase in IL-5 expression was measured after irradiated MARV stimulation.

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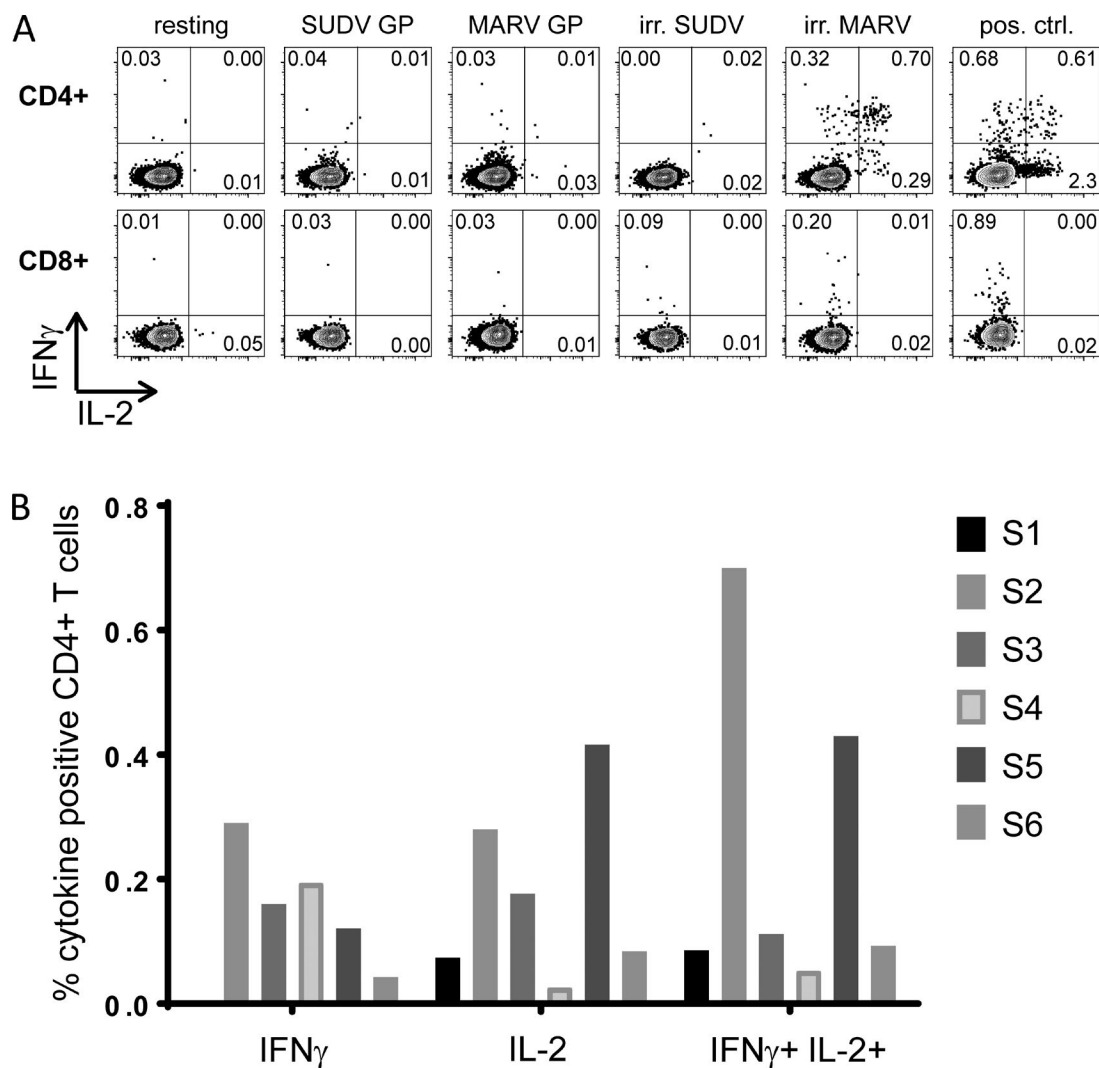Adaptive immune responses in Marburg virus survivors | Stonier et al.

Figure 1. **Analysis of MARV survivor T cell responses in whole blood cultures.** Heparinized whole blood samples were collected from MARV survivors ($n = 6$) ~9 mo after the 2012 MARV outbreak in Uganda. (A) Representative IFN-γ and IL-2 responses in CD4$^+$ and CD8$^+$ T cells to recombinant SUDV GP or MARV GP, irradiated SUDV or MARV, and staphylococcus enterotoxin B for 22 h are shown. (B) The frequency of IFN-γ single-positive, IL-2 single-positive, or IFN-γ and IL-2 double-positive responses among total CD4$^+$ T cells are shown for six MARV survivors.

CD40L expression and cytokine responses

Although we demonstrated the utility of whole blood cultures to measure T cell responses in MARV survivors (Fig. 1), the use of PBMCs allows for the analysis of greater cell numbers. Additionally, purified PBMCs can better elaborate cytokine responses in comparison to whole blood cultures (Hoffmeister et al., 2003). We therefore developed an assay to use PBMCs instead of whole blood to attempt a more detailed and comprehensive analysis of T cell responses in MARV survivors. PBMCs were cultured with the specific antigens as before, except that monensin (instead of brefeldin A) was added after 2 h of culture to begin trapping intracellular cytokines. Additionally, fluorescently labeled antibodies against CD107a and CD40L were added at this time to enable detection of a degranulation phenotype (Betts et al., 2003) and CD4$^+$ T cell

activation (Chattopadhyay et al., 2006), respectively. Cultures were then incubated for a further 16 h. Cell viability gated on lymphocytes after culture (18 h total; 16 h with monensin) was measured by an amine-reactive dye and found to be 91 ± 5.8% inclusive of all survivors and culture conditions (unpublished data).

With a combination of CD40L and IFN-γ expression, we identified activated CD4$^+$ T cells after stimulation with irradiated MARV antigen from seven MARV survivors from the 2012 outbreak. In our previous collection, we had only obtained samples from six survivors; however, in the intervening time, we gained access to an additional survivor sample for flow cytometry analysis. IFN-γ expression was coordinately expressed with CD40L, which is consistent with the description of CD40L expression as an activation marker
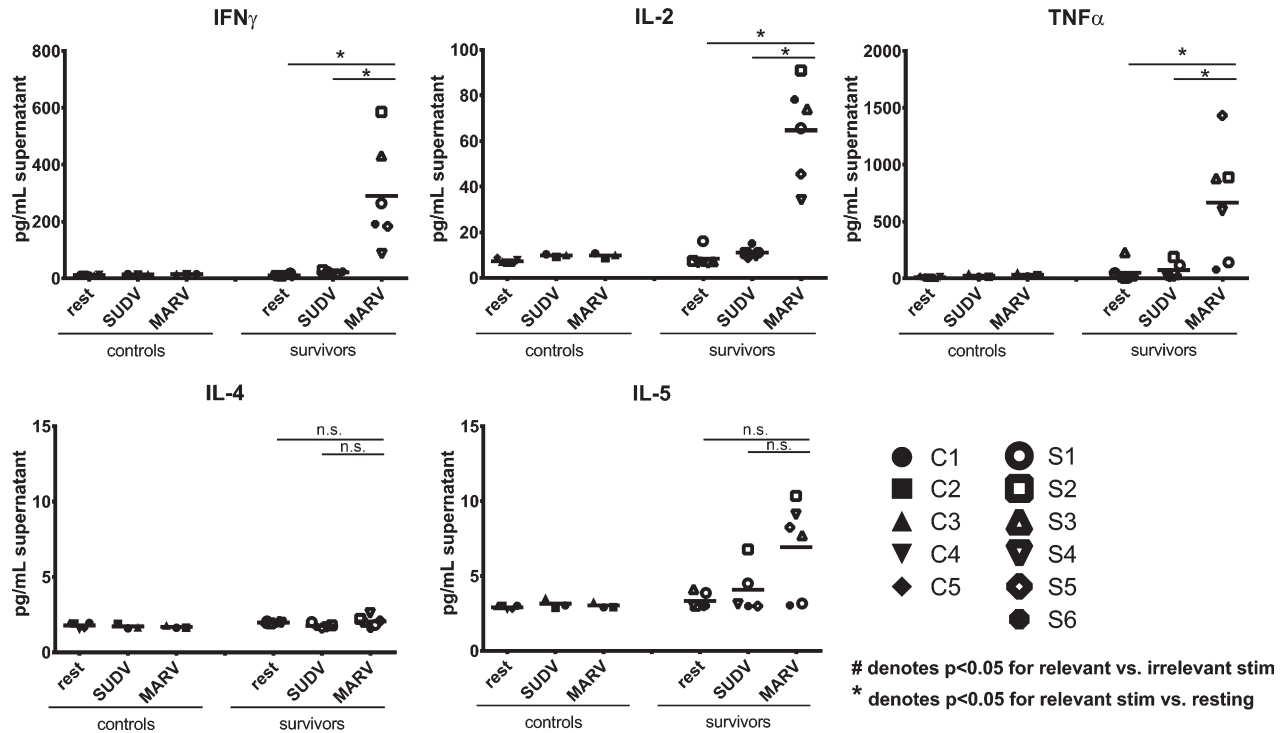
Figure 2. **Multiplex ELISA for secreted cytokines.** Supernatant was collected after 22-h stimulation of whole blood cultures from MARV survivors ($n = 6$) and uninfected controls ($n = 5$). Supernatants were analyzed in duplicate. Mean values for the indicated cytokine secretion are reported among survivor and control populations. * indicates $P < 0.05$ for irradiated MARV versus irradiated SUDV stimulations. # indicates $P < 0.05$ for irradiated MARV stimulation versus resting cultures. n.s. indicates no significant difference.

(Fig. 3 A, top; Chattopadhyay et al., 2006). TNF and IL-2 expression also followed the same pattern with regard to CD40L expression (not depicted). PBMCs from uninfected controls demonstrated no cytokine response to the irradiated MARV antigen (Fig. 3 A, middle). Representative IFN-γ and TNF staining, gated on CD40L$^+$ CD4$^+$ T cells, is depicted in Fig. 3 A (bottom) for all survivors after irradiated MARV antigen stimulation.

To measure the overall magnitude of the CD4$^+$ T cell response, we used a Boolean gating strategy to determine the frequency of IFN-γ−, TNF-, or IL-2–positive events that were identified by CD40L expression after stimulation (Fig. 3 B). CD40L expression has been shown to increase in a nonspecific manner during culture (Chattopadhyay et al., 2006), and this analysis enabled us to determine the frequency of only cytokine-expressing CD40L$^+$ CD4$^+$ T cells to give a more accurate representation of the specific response. Resting, SUDV GP, and irradiated SUDV antigen cultures elicited negligible CD40L$^+$ cytokine$^+$ responses (Fig. 3 B), reinforcing both the utility and specificity of the stimulation and analysis. Furthermore, PBMC samples from uninfected controls exhibited no coordinate expression of CD40L and IFN-γ after stimulation with irradiated MARV, demonstrating that this response is specific to MARV survivors. MARV GP elicited responses of low magnitude, whereas the CD40L$^+$

cytokine$^+$ response to irradiated MARV was more robust in comparison (Fig. 3 B). CD4$^+$ T cell responses directed against MARV GP were low in magnitude compared with irradiated virus, as in Fig. 1, despite skewing the amount of antigen toward favoring GP-specific responses. The composition of each individual survivor's CD40L$^+$ response to irradiated MARV stimulation in terms of IFN-γ, TNF, and IL-2 is depicted in Fig. 3 C. Resting culture values were subtracted to account for any background cytokine expression. The most dominant response was found to be cells producing IFN-γ, TNF, and IL-2, whereas the double- and single-positive responses for these cytokines varied to a greater extent among survivors (Fig. 3 C).

We had measured CD8$^+$ T cell responses in whole blood cultures previously (Fig. 1 and not depicted), but the IFN-γ and IL-2 cytokine responses were low in magnitude. We considered it possible that perhaps these parameters were not capturing the totality of the CD8$^+$ T cell response. Therefore, we incorporated CD107a staining into our analysis to detect any CD8$^+$ T cells that had degranulated and/ or produced cytokines in response to MARV antigens. To this end, we analyzed CD107a and IFN-γ expression after MARV stimulation. Control CD8$^+$ T cells demonstrated no detectable IFN-γ expression in response to MARV stimulation, whereas CD107a expression was found in both resting
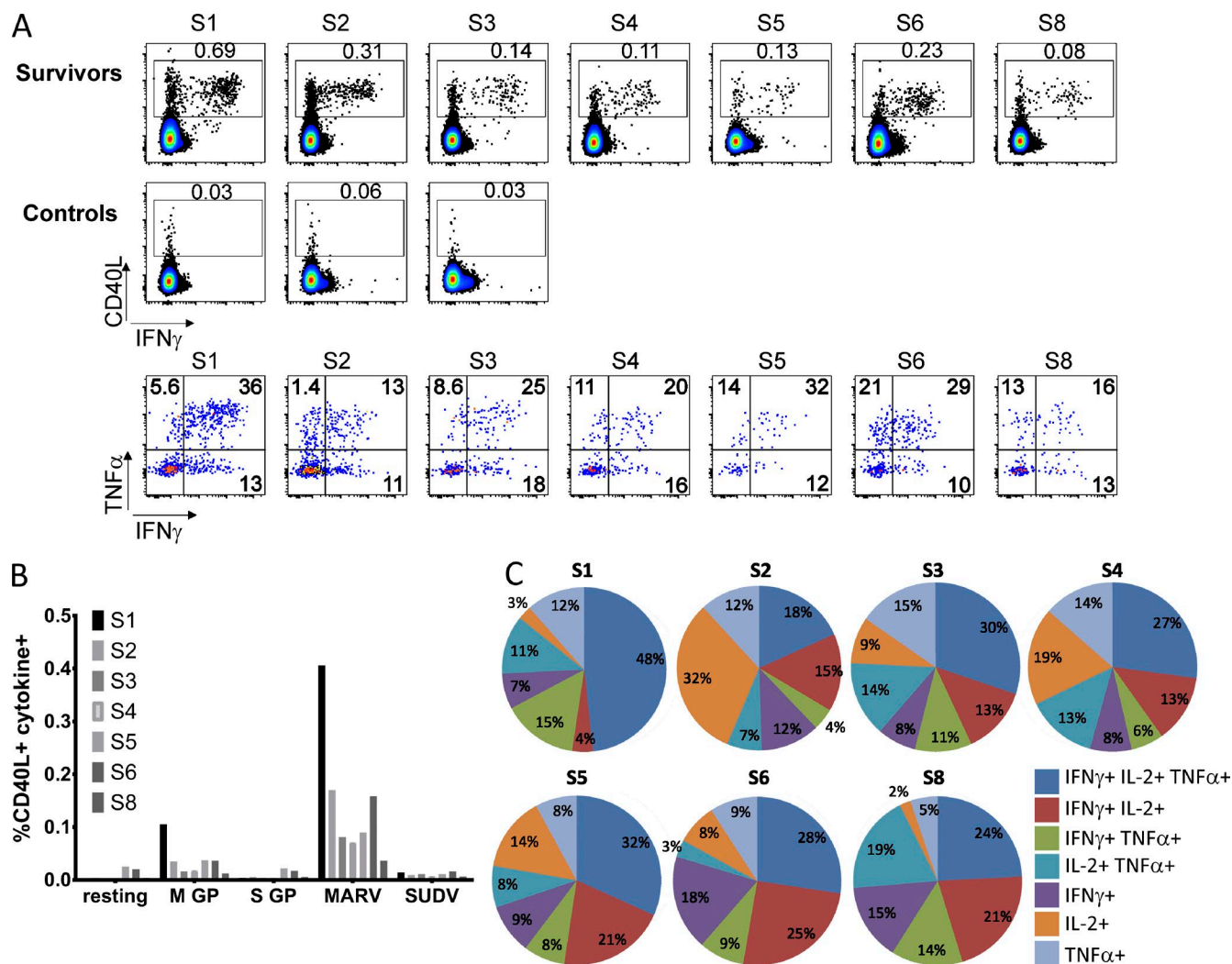
Figure 3. Flow cytometry analysis of MARV survivor CD4+ T cell responses in PBMC cultures. Purified PBMCs from MARV survivors (*n* = 7) and uninfected controls (*n* = 3) were collected 27 mo after the MARV outbreak and stimulated with antigens as before for a total of 18 h. Monensin, CD40L antibody, and CD107a antibody were added after 2 h. (A) CD40L and IFN-γ staining on CD4+ T cells after stimulation with irradiated MARV antigen. Top panels depict responses seen in survivors, and uninfected control responses are depicted in the middle panel. The bottom panel demonstrates IFN-γ and TNF staining after gating on CD40L+ CD4+ T cells from survivors as identified in the top panel. (B) Bar graph shows the frequency of total cytokine+ CD40L+ CD4+ T cells after stimulation with the indicated MARV and SUDV antigens. (C) Pie charts display the composition of the CD40L+ cytokine response in MARV survivors after stimulation with irradiated MARV.

and MARV-stimulated cultures. This apparently nonspecific CD107a expression was found in resting cultures for survivors' CD8+ T cells as well. CD107a expression in the context of IFN-γ and TNF (not depicted) demonstrated specificity with regard to MARV stimulation, and only in a subset of survivors (Fig. 4, right panels). These results confirm our earlier findings showing little to no CD8+ T cell responses in MARV survivors and add the additional functional characteristic of apparent cytotoxic responses.

CD4+ T cell responses to MARV were readily detectable in many cases, either in whole blood samples or purified PBMCs (Figs. 1 and 3). In contrast, CD8+ T cell responses were far more rare (Figs. 1 and 4), regardless of the sample type. Al-

though the CD8+ T cell response to EBOV is relatively well documented in survivors and nonsurvivors during infection or shortly after convalescence (McElroy et al., 2015; Agrati et al., 2016; Ruibal et al., 2016), still little is known about the long-term persistence of these responses. We demonstrate here that anti-MARV CD4+ T cell responses are present for at least 2 yr after infection. Furthermore, SUDV survivors' CD4+ T cell responses have been shown to persist for more than a decade, whereas CD8+ T cell responses were far more rare (Sobarzo et al., 2016). Blood samples collected at the time of this SUDV outbreak demonstrated elevated CD8+ T cell counts in nonfatal cases (Sanchez et al., 2004), which suggests there was an active CD8+ T cell response that had diminished
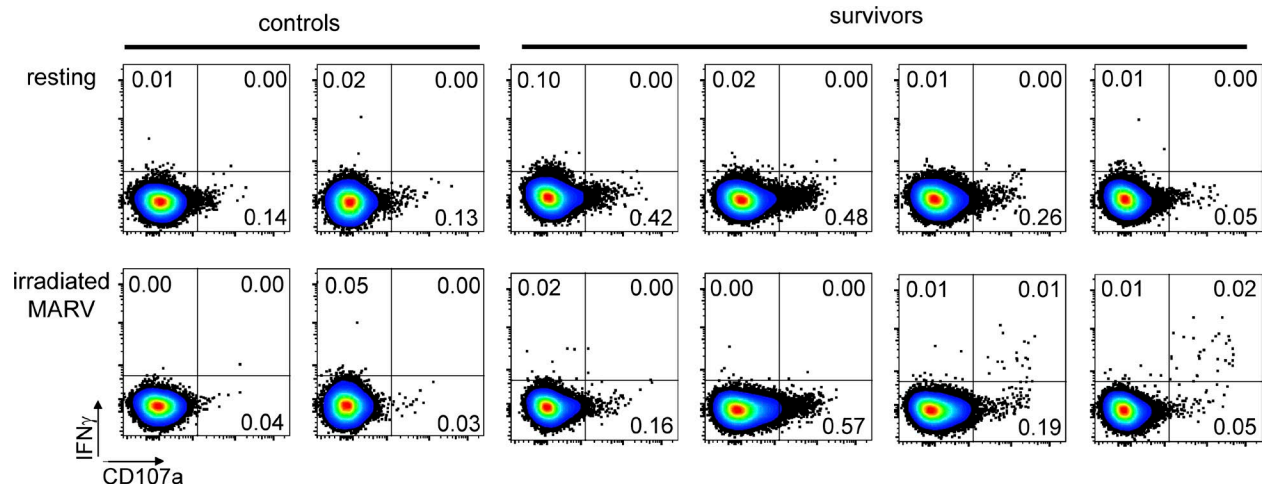
Figure 4. MARV survivor CD8+ T cell responses. PBMCs from MARV survivors (*n* = 4) and uninfected controls (*n* = 2) were cultured as before. Plots depict IFN-γ+ and CD107a+ CD8+ T cell responses after stimulation with irradiated MARV. Left panels depict staining of uninfected control samples. Right panels show representative staining of four MARV survivors with and without apparent CD8+ T cell responses. Positive CD8+ T cell responses are considered to be CD107a+ IFN-γ+.

greatly over time. A similar phenomenon may have occurred with the MARV survivor immune responses we have reported here, albeit over a shorter timescale. Indeed, a study of immune response in smallpox vaccine recipients showed that CD8+ T cell responses measured longitudinally diminished greatly over time and were present at very low frequencies when measured 2 yr after vaccination (Miller et al., 2008). Alternatively, CD4+ T cell cytokine responses were found to be of relatively lower magnitude compared with CD8+ T cell cytokine responses in EBOV survivors when assayed between 28 and 144 d after onset of symptoms (McElroy et al., 2015). MARV and SUDV survivor immune responses, however, appear to heavily favor CD4+ T cell responses, though these studies were conducted with samples collected months and years after convalescence. Despite the difference in time frames, the MARV survivor CD4+ T cell cytokine responses we report in this study are of greater magnitude than those observed with EBOV survivors. These discordant immune responses among filovirus infection survivors may indicate that the composition of T cell responses to filovirus infection may vary significantly between MARV, EBOV, and SUDV.

Antiviral antibody responses

To address the humoral immune response to MARV infection, we collected serum from uninfected control donors and survivors and analyzed these samples for IgG antibodies against irradiated MARV antigen. Serum samples were serially diluted to determine an end titer. All survivors developed IgG responses to irradiated MARV, reaching an end titer between 4.25 and 6 (LOG$_{10}$ serum dilutions; Fig. 5 A). Cell lysates expressing various MARV proteins were used to determine the individual protein specificity of the MARV IgG response. Responses to lysate-derived antigens were denoted

as + or − based on a signal-to-noise ratio of cell lysates without MARV proteins (Table S1). All survivors were found to have IgG responses against MARV nucleoprotein (NP) and GP, but not against VP35 or VP24 (Table S1). Survivors 2–7 had IgG responses to VP40, and survivors 1–6 had responses to VP30. Control sera were also included in these analyses and found to be nonreactive against lysates bearing MARV proteins (unpublished data). Antibody reactivity to MARV proteins is not likely to strictly be a consequence of relative protein abundance in the virion, as VP30 and VP24 constitute a similar fraction of overall virion protein content (Kiley et al., 1988). However, MARV VP40 is roughly twice as abundant as VP35 in the MARV virion (Kiley et al., 1988), and both are described to be IFN antagonists (Valmas et al., 2010; Guito et al., 2017). Antibody reactivity in MARV survivor serum to VP40 and not VP35 may be reflective of either greater accessibility to VP40 because of its greater abundance or its association with the viral membrane. Though it is beyond the scope of our current studies, it would be intriguing to determine whether antibodies to VP40 can inhibit its IFN antagonism and/or its role in virion assembly, which could impute novel roles for antibody function in filovirus infections.

To address one potential function of the MARV-specific serum antibodies, we used a plaque reduction/neutralization test (PRNT) to determine whether survivor serum could neutralize live virus in vitro. Serum samples were serially diluted beginning at 1:10 and preincubated with MARV. This mixture was then used to inoculate Vero E6 cells, and resulting plaques were counted. Serum from only two survivors, S2 and S3, neutralized MARV plaque formation by at least 50%, our predetermined threshold (Fig. 4 B). Uninfected control serum samples had very low neutralization values, illustrating the specificity of this response in MARV survivors. We evalu-
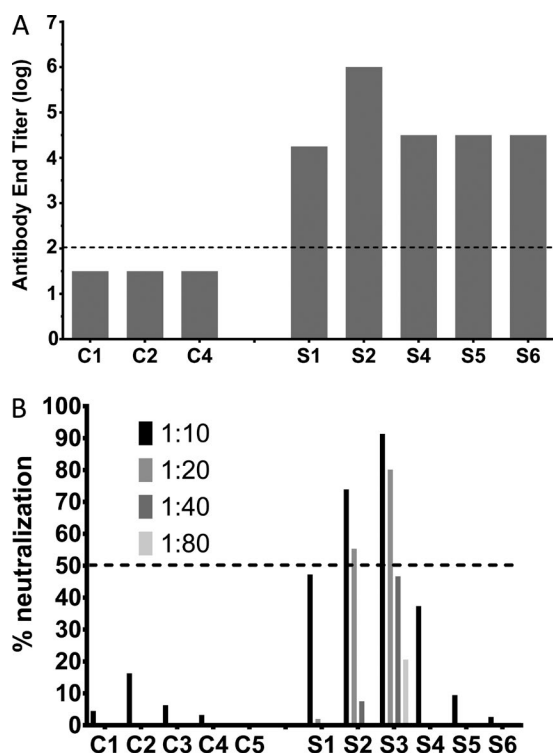
Figure 5. Antibody responses from MARV survivors. (A) Irradiated MARV was coated on plates to capture MARV-specific antibodies from serum samples collected from MARV survivors ($n = 5$) and uninfected controls ($n = 3$). Total IgG was detected using an anti–human IgG-HRP antibody and ABTS substrate. End titer is reported as the antilog of the reciprocal of the last dilution of serum that exceeds a threshold based on naive serum. The limit of detection is depicted by the dashed line. (B) Beginning at 1:10, serial 1:2 dilutions of serum samples from MARV survivors ($n = 6$) and uninfected controls ($n = 5$) were incubated with MARV before inoculation of Vero E6 cells. The percentage of neutralization is reported at 1:10, 1:20, 1:40, and 1:80 dilutions based on the reduction of plaques relative to control MARV-infected Vero cells. Dashed line indicates 50% neutralization, or $PRNT_{50}$, which is used to define positive neutralizing responses. Serum samples used in ELISA and PRNT were analyzed in duplicate.

ated serological responses longitudinally with serum samples collected every 6 mo. For our purposes, we used a threshold of $\geq 50\%$ neutralization of MARV ($PRNT_{50}$) to determine positive neutralization responses. For samples collected 9 and 15 mo after the outbreak, two out of six survivors had neutralizing antibody titers between 1:20 and 1:40 (Fig. 3 B and Table S2). Neutralizing responses to MARV began to diminish 21 mo after the outbreak and dropped below our threshold after 27 mo. In contrast, antibody titers to irradiated MARV over this same time frame remained consistent with no drop in antibody end titer (Table S2).

Whereas our current knowledge of T cell responses to various filovirus infections suggests common themes, such as robust Th1-skewed $CD4^+$ T cell responses, the antibody responses appear to be more divergent. MARV survivors generate IgG responses against GP, NP (all survivors), VP40 (S2–7),

and VP30 (S1–4, 6, and 7) but not to VP35 or VP24 (Table S1). These profiles of viral protein reactivity resemble the serological profile of SUDV survivors (Sobarzo et al., 2013). Neutralizing antibody responses in MARV survivors, however, appear to be more divergent from that seen in SUDV survivors. Analyses of survivors of the SUDV outbreak in Gulu, Uganda, demonstrate long-lived neutralizing antibody titers (Sobarzo et al., 2013). Admittedly, this particular MARV cohort is small in number ($n = 8$); however, in a similar sample size from a recent SUDV outbreak in Kibaale, Uganda (Albariño et al., 2013), five out of five survivors had neutralizing serum responses (Sobarzo et al., 2015). Even more striking is the magnitude of neutralizing titers among recent SUDV survivors: three out of five had $PRNT_{50}$ values $\geq 1/80$. Neutralizing antibody responses in a MARV survivor have been previously reported (Flyak et al., 2015), but our data are the first longitudinal analyses to demonstrate a decline in these responses, despite maintaining high antibody titers overall. Our first serum samples were obtained ~9 mo after the outbreak, so it remains possible that all survivors may have had neutralizing responses at time points more proximal to infection. What is clear, however, is that these responses are lower in magnitude than analogous SUDV survivors (Sobarzo et al., 2015).

Neutralizing antibody responses have been achieved through vaccination against MARV GP in mouse, guinea pig, and cynomolgus macaques (Shedlock et al., 2013; Grant-Klein et al., 2015). Interestingly, cynomolgus macaques vaccinated against MARV GP showed diminishing neutralizing antibody titers over time (Mire et al., 2014), similar to our observations with human survivors of MARV infection. It would be interesting to see whether a similar phenomenon would occur in any surviving naive macaques that were experimentally infected with MARV. Whereas neutralizing antibodies elicited by vaccination against filoviruses is a coveted immune response, functions of nonneutralizing antibodies have been described in other viral immune responses. Nonneutralizing antibodies to HIV and lymphocytic choriomeningitis virus GPs inhibit infection of DCs and macrophages (Holl et al., 2006) and limit virus spread (Hangartner et al., 2006), respectively. Various nonneutralizing functions of antibodies elicited by vaccination against HIV have been described previously in great detail (Chung et al., 2014, 2015). Perhaps more intriguing is a study showing that nonneutralizing antibodies elicited by vaccination against influenza NP can play a role in aiding the T cell response in protecting mice against influenza infection (Carragher et al., 2008). Indeed, immunization with influenza–NP antibody complexes elicited IFN-γ production from $CD8^+$ and $CD4^+$ T cells (Zheng et al., 2007), indicating a Th1-skewed immune response. Because all the MARV survivors in this have antibodies recognizing MARV NP (Table S1), these studies describing a role for NP-specific antibodies in T cell responses may provide a roadmap for the ontogeny of the MARV survivor immune responses described herein.

Our studies are the first to provide a detailed longitudinal analysis of immune responses among human MARV

survivors. Our findings highlight that although CD4^{+} T cell responses may be common among human filovirus survivors, the neutralizing antibody response varies to a greater degree. The discord in neutralizing responses between MARV and SUDV survivors indicates that there is a critical knowledge gap regarding what can be considered a protective response to filovirus infection.

MATERIALS AND METHODS

Study design

Subjects included confirmed survivors, according to patient PCR and ELISA results, from the MARV outbreak of 2012 in the Ibanda and Kabale districts of Uganda, as well as healthy local community members that were not infected. Based on records obtained from the Centers for Disease Control in Uganda, we identified a total of 10 survivors from this outbreak. We were able to collect from eight of these survivors, with the others not being available or too young in age for collections. Whole blood stimulations were conducted with blood samples from six survivors, and PBMC stimulations were conducted with samples from seven survivors. Survivors in these studies ranged in age from 18 to 50 yr. Whole blood analyses involved two male and four female survivors, and PBMC analyses included three male and four female survivors. An additional survivor (S7, female) for whom we have serological data but not flow cytometry data is included in Tables S1 and S2. Health questionnaires administered before sample collection indicated that the study subjects were relatively healthy, with a few individuals reporting nonspecific malaise. One survivor was receiving medication for malaria.

Ethics statement

The study was approved by the Helsinki committees of the Uganda Virus Research Institute in Entebbe, Uganda (reference number GC/127/13/01/15); Soroka Hospital, Beer-sheva, Israel (protocol number 0263–13-SOR); and the Ugandan National Council for Science and Technology (registration number HS1332). Written informed consent as well as a personal health questionnaire was completed for each subject.

Flow cytometry assays

Whole blood cultures were established as previously reported (Sobarzo et al., 2016). In brief, 0.25 ml of fresh whole blood was mixed with 0.75 ml RPMI medium and 5% FBS, and cultures were incubated with 50 μg MARV or SUDV GPs or 10 μg of noninfectious irradiated whole virus preparations of MARV (MARV/*Homo sapiens*–tc/DEU/1967/Hesse-Ci67) or SUDV (SUDV/*Homo sapiens*–tc/UGA/2000/Gulu). The amount of antigen used for stimulation was titrated using survivor samples from an unrelated SUDV outbreak in 2000-2001. In these studies, cultures were supplemented after 18 h with brefeldin A and incubated for a further 4 h. After 22 h total, cultures were vortexed, cells were pelleted, and the resultant supernatant was collected. Cells were subjected

to two 5-min incubations with a Tris–ammonium chloride solution to lyse red blood cells and then subsequently stained for surface and intracellular antigens. This approach enabled a flow cytometry analysis of cytokine response with matched supernatants for an expanded cytokine analysis by ELISA. PBMCs were collected in cell preparation tube vacutainers (BD Biosciences) and isolated according to the manufacturer's protocol. Total cell yields were split between various culture conditions in RPMI medium + 5% FBS: no stimulation, 50 μg recombinant MARV or SUDV GP, 10 μg of irradiated MARV or SUDV, or 1 μg staphylococcus enterotoxin B. Culture volume across all conditions was 1 ml. After 2 h, cultures were supplemented with monensin and antibodies against CD40L and CD107a. Total culture time was 18 h. After stimulation, cells were stained with the amine-reactive Aqua dye (Thermo Fisher) to detect dead cells, nonspecific staining was blocked with 1% mouse serum, and surface proteins were stained with fluorescently labeled antibodies. After fixation and permeabilization, intracellular cytokines were detected. Samples were acquired on an LSR II flow cytometer (BD Biosciences) at the Uganda Virus Research Institute/Medical Research Council facilities in Entebbe, Uganda. Flowjo (version X; Treestar) was used to analyze flow cytometry data. Antibodies used in these studies are as follows: CD3 (S4.1/Qdot 605 for whole blood and UCHT1/BV650 for PBMCs), CD4 (S3.5/Qdot 655 for whole blood and RPA–T4/BV605 for PBMCs), CD8 (3B5/Qdot 705 for whole blood and SK1/BV711 for PBMCs), CD40L (TRAP1/PE), CD107a (eBioH4A3/eFluor660), IFN-γ (4S.B3/APC-eFluor780), IL-2 (MQ1-17H12/PE-Cy7), and TNF (MAb11/FITC).

PRNT

PRNTs were performed as previously described (Sobarzo et al., 2016). Neutralization titers were determined to be the last dilution of serum that reduced the number of plaques by 50% compared with control wells. Plaque reduction neutralization assays were performed in the BSL-4 laboratory of the United States Army Medical Research Institute of Infectious Diseases.

Cytokine and chemokine detection using Q-Plex ELISA–based chemiluminescent assay

Levels of human cytokines were measured in whole blood culture supernatants using Q-Plex technology (Quansys Biosciences) according to the manufacturer's instructions. Readouts were obtained with a Quansys Imager (Quansys Biosciences), and results were analyzed using the Q-View Software program (Quansys Biosciences).

ELISA antigens

For ELISA assays, irradiated MARV (Ci67 isolate), recombinant MARV GP$_{1-649}$, and total 293T cell lysate that expressed a given recombinant MARV protein (NP, VP24, and VP35) were used as the capture antigens. Total IgG was detected with an anti–human IgG antibody conjugated to HRP. 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)

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(KPL) was used as the substrate for irradiated MARV end titer ELISAs, and a chemiluminescent substrate was used in ELISAs for viral proteins.

Statistical analysis

Statistical analyses were performed using Prism software (version 6.01; GraphPad). Correlation analysis was assessed by the Spearman nonparametric test. Differences in cytokine values between study groups were assessed by ANOVA and Wilcoxon rank sum test; p-values represent two-sided p-values, and p-values <0.05 were considered statistically significant.

Online supplemental material

Table S1 shows MARV survivor serum reactivity to individual MARV proteins. Table S2 shows a longitudinal analysis of anti–MARV serum antibody responses by ELISA and PRNT.

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SUPPLEMENTAL MATERIAL

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Table S1.   **MARV survivor serum reactivity to individual virus proteins**

Survivor	GP	NP	VP35	VP24	VP40	VP30	Whole virus
S1	+	+	−	−	−	+	+
S2	+	+	−	−	+	+	+
S3	+	+	−	−	+	+	+
S4	+	+	−	−	+	+	+
S5	+	+	−	−	−	−	+
S6	+	+	−	−	+	+	+
S7	+	+	−	−	+	+	+
S8	+	+	−	−	+	−	+

MARV proteins were expressed in 293T cells, and cell lysates were used as ELISA capture antigens. Antibody responses were scored as positive or negative based on the signal-to-noise ratio of MARV protein-transfected lysates and nontransfected lysates.

Table S2.   **Longitudinal analysis of antibody responses**

Survivor		Time of collection after outbreak				
		9 mo	15 mo	21 mo	27 mo	33 mo
S1	$PRNT_{50}$	<10	<10	<10	<10	<10
	End titer	4.25	4	4	3.75	4
S2	$PRNT_{50}$	20	20	10	<10	<10
	End titer	6	5.75	5.5	5.5	6
S3	$PRNT_{50}$	40	40	n.d.	<10	<10
	End titer	5.5	5.0	5.5	6	5.5
S4	$PRNT_{50}$	<10	10	<10	<10	<10
	End titer	4.5	4.5	4.25	4.0	4.5
S5	$PRNT_{50}$	<10	<10	<10	<10	<10
	End titer	4.5	4	4.5	4.5	4.5
S6	$PRNT_{50}$	<10	<10	<10	<10	<10
	End titer	4.5	4.5	4.75	5.5	6
S7	$PRNT_{50}$	n.d.	10	n.d.	n.d.	n.d.
	End titer	n.d.	4.5	n.d.	n.d.	n.d.
S8	$PRNT_{50}$	n.d.	<10	<10	<10	<10
	End titer	n.d.	4.5	n.d.	4.75	4.75

Antibody end titer and $PRNT_{50}$ titers are shown at 6-mo intervals beginning 9 mo after the end of the 2012 MARV outbreak in Uganda. n.d. indicates that a value was not determined because of sample unavailability. All serum samples were analyzed in duplicate.