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Research paper

Multiple viral proteins and immune response pathways act to generate robust long-term immunity in Sudan virus survivors

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ABSTRACT

Background: Profiles of immunity developed in filovirus patients and survivors have begun to shed light on antigen-specific cellular immune responses that had been previously under-studied. However, our knowledge of the breadth and length of those responses and the viral targets which mediate long-term memory immunity still lags significantly behind.
Methods: We characterized antigen-specific immune responses in whole blood samples of fifteen years post-infected survivors of the Sudan virus (SUDV) outbreak in Gulu, Uganda (2000−2001). We examined T cell and IgG responses against SUDV complete antigen and four SUDV proteins; glycoprotein (GP), nucleoprotein (NP), and viral protein 30 (VP30), and 40 (VP40).
Findings: We found survivors-maintained antigen-specific CD4+ T cell memory immune responses mediated mainly by the viral protein NP. In contrast, activated CD8+ T cell responses were nearly absent in SUDV survivors, regardless of the stimulating antigen used. Analysis of anti-viral humoral immunity revealed antigen-specific IgG antibodies against SUDV and SUDV proteins. Survivor IgGs mediated live SUDV neutralization in vitro and FcγRI and FcγRIII antibody Fc-dependent responses, mainly via antibodies to the viral proteins GP and VP40.
Interpretation: We highlight the key role of several proteins, i.e., GP, NP, and VP40, to act as mediators of distinctive and sustained cellular memory immune responses in long-term SUDV survivors. We suggest that the inclusion of these viral proteins in vaccine development may best mimic survivor native memory immune responses with the potential of protecting against viral infection.
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1. Introduction

Ebola virus belongs to the *Filovirus* family of the *Mononegavirales*, a large order of enveloped viruses with non-segmented, negative-strand (NNS) RNA genomes [1]. From 3′ to 5′ end, the genome encodes for seven structural proteins: a nucleoprotein (NP), viral proteins 35 (VP35) and VP40, a glycoprotein (GP), secreted GP (sGP), VP30 and VP24, and the non-structural viral RNA-dependent RNA polymerase (L) [1,2].

In humans, Ebola virus causes a hemorrhagic fever disease (EVD) with high morbidity and mortality [2]. Of the five members of the genus, the Zaire ebolavirus (EBOV) and Sudan ebolavirus (SUDV) species pose the greatest threat to humans [3]. They have been the prime cause of dozens of periodic outbreaks across Africa. Cases number in the thousands and the associated case-fatality ratio is 30–90% [4,5].

Profiles of immunity developed in *Filovirus* survivors have recently begun to shed light on immune responses that had been understudied [6–10]. Important immune mediators of survival, during acute and early convalescent stages, are also being determined [6,7,11].

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Research in context

Evidence before this study

We searched PubMed without language restrictions using the terms "Ebola virus" or "filovirus" combined with both "immune persistence" and "human survivors" for articles published up to April 1, 2019. Previous studies in humans have indicated that recovery is largely dependent upon, and associated with, the development of cell-mediated and humoral immunity. Evidence from cohorts of EVD survivors from the 2014 West Africa epidemics indicated a pivotal role for antigen-specific CD8+ T cell responses in the control of viral replication and disease outcome. These responses were still detected up to two-years post infection. In contrast, studies of long term SUDV and MARV cohorts of survivors demonstrated potent virus-specific CD4+ T cell responses. We need to understand better the relative roles of the different cellular immune responses and their viral antigen mediators in eliciting long-term memory immunity in Filovirus survivors.

The added value of this study

Our study provides new evidence concerning the relative roles of specific viral antigen (GP, NP, and VP40) drivers of cellular and humoral memory immunity in long-term SUDV survivors. The immune responses included robust CD4+ T cell memory immunity and IgG antibodies capable of mediating both in vitro neutralization and antibody Fc-dependent responses. Our results also highlight a strong association between cellular and humoral memory responses in SUDV survivors.

Implications of all the available evidence

The recent devastating Ebola virus outbreaks have highlighted the urgent need for an effective and long-lasting vaccine. Preliminary results from vaccine trials in human are promising. However, the current advanced candidate vaccines are based solely on Ebola virus glycoprotein. Their capacity to confer sustained humoral and cellular immune responses is uncertain. Our cohort of long-term SUDV survivors provided a unique opportunity to study the profile of naturally-acquired immune memory responses years after recovery. The data we collected from this cohort of survivors indicate that several antigen-specific immune memory responses may play a vital role in providing long-lasting protection - which could be similar in other filovirus survivors. We propose that the inclusion of these additional viral proteins (i.e. NP and VP40) will benefit vaccine design.

Studies of the pathogenesis of EVD showed that recovery is largely dependent on, and associated with, the development of both cell-mediated and humoral immune responses [3,12]. Recent works on cohorts of EVD patients from the 2014 West Africa epidemics demonstrated that activated antigen-specific T cell responses during the acute stage of infection correlated with favorable clinical outcome [6–8,11]. Cellular immunity in these patients was shown to be associated with significant antigen-specific CD8+ T cell responses [6,7,9,11]. In contrast, data collected, from SUDV and MARV cohort of survivors, several years post infection, demonstrated potent virus-specific CD4+ T cell responses [10,13]. Although indicative, our understanding of the specific viral targets, immune components and pathways that drive a sustained cellular mediated memory immune response for years after Ebola virus infection is still vague [12,14].

Considering the threat these viruses continue to pose; several EBOV vaccines are under development. Of these, two hybrid recombinant viruses incorporating the immunogenic GP are most advanced [15,16]. These vaccines induce GP-specific humoral immunity; however, their ability to trigger long-term T cell responses following vaccination is still unclear [16–18]. It was suggested that any vaccine used for active immunization of disease contacts should optimally induce protection that lasts at least two years and ideally up to ten years [18,19]. In this regard, studies of residual immune responses in naturally recovered survivors are likely to improve our understanding of Ebola virus correlates of protection, which will help guide vaccine development [14,15]. Towards improving our understanding of recovery from and long-term immunity to *Filoviruses*, we studied antigen-specific immune responses in long-term survivors of the 2000–2001 SUDV outbreak in Gulu, Uganda. T cell responses and IgG functionality were analyzed in stimulated PBMCs and matched sera samples against irradiated SUDV and four selected SUDV individual proteins.

2. Materials and methods

2.1. Study design

Subjects were recruited with the assistance of the Uganda Virus Research Institute (UVRI). All survivors had a documented clinical history of SUDV infection according to their PCR and ELISA results during the SUDV outbreak of 2000–2001 in Gulu district, Uganda [20]. Fifteen individual survivors and three healthy local community members that were not infected, which served as controls, participated in this study. To monitor antigen-specificity of immune responses, we isolated irradiated SUDV complete virus (gulu isolate) and expressed and purified four of the eight gulu isolate proteins: GP, NP, VP30 and VP40 [21]. These were selected based on recent works in EBOV patients and our previous experience with this cohort of survivors [7,11,13]. Cellular immune response controls included a positive antigen control, Staphylococcus enterotoxin B (SEB), and negative antigen controls, irradiated Marburg virus (MARV) and MARV GP. T cell immunity was monitored by stimulating PBMC with one of the antigens and then assessing the response by flow cytometry and multiplex ELISA [10,13]. Persistence of antigen-specific IgGs was measured by ELISA [22]. IgG functionality was measured by virus neutralization in a plaque reduction neutralization assay (PRNT) using infectious SUDV [23], and by antibody Fc-dependent reporter assay [24].

2.2. Ethics statement

The study was approved by the Helsinki committees of the UVRI 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 (UNCST) (registration number HS1332). Written informed consent, as well as a personal health questionnaire, was completed for each subject participated in this study. Study participants were all adults and not related. We confirm that all experiments were performed in accordance with the relevant guidelines and regulations.

2.3. Antigens and stimulations

PBMCs stimulation antigens included irradiated sucrose gradient-purified, SUDV-gulu isolate (Sudan virus/H.sapiens-tc/UGA/2000/Gulu) [13], and His-tagged (His_6) purified SUDV recombinant proteins GP ($GP_{1-649,}$ representing the first 649 amino acids) [13], NP, VP30 and VP40 [21]. Control stimulation included a Staphylococcus Enterotoxin B (SEB) (Sigma-Aldrich,), an irradiated whole virus preparation of Marburg virus (Marburgvirus/H.sapienstc/DEU/1967/Hesse-Ci67), His-tagged (His_6) purified recombinant proteins, Marburg viral proteins GP [10]. For ELISA assays, irradiated SUDV, and SUDV proteins GP, NP, VP30, and VP40 were used as the capture antigens.

2.4. Flow cytometry

Flow cytometry stimulation assays were described elsewhere [10,13]. Briefly, PBMCs from survivors and noninfected controls were collected in CPT vacutainers (BD Biosciences) and isolated according to the manufacturer's protocol. Total cell yields were split between various culture conditions in RPMI+5%FBS: no stimulation (background), 10 μg of irradiated SUDV and SUDV proteins GP, NP, VP30, and VP40. SEB at 1 μg concentration was used as a positive control, and 10 μg of irradiated MARV, and MARV GP were used as negative controls. Culture volumes across all conditions were 1 ml. After 2 h incubation, PBMC-stimulated cultures were supplemented with monensin and antibodies against CD40L and CD107a. CD107a and CD40L markers were targeted to enable detection of a degranulation phenotype [25] and CD4+ T cell activation [26] respectively. Total culture time was 18 h. Following incubation, cells were stained with the amine-reactive Aqua dye (Thermo Fisher) to detect dead cells, nonspecific staining was blocked with 1% mouse serum (Thermo Fisher), and surface proteins were stained with fluorochrome-labelled antibodies. After fixation and permeabilization, intracellular cytokines were detected. Samples were acquired on an LSRII (BD Biosciences) at the MRC/UVRI facilities in Entebbe, Uganda and analyzed with FlowJo software (version X, TreeStar). Antibodies used in these studies are as follows: CD3 (UCHT1/BV650), CD4 (RPA-T4/BV605), CD8 (SK1/BV711), CD40L (TRAP1/PE), CD107a (eBioH4A3/eFluor660), IFN-γ (4S. B3/APC-eFluor780), IL-2 (MQ1-17H12/PE-Cy7) and TNF (MAb11/FITC).

2.5. Cytokine and chemokines multiplex ELISA

The levels of secreted cytokines and chemokines were analyzed as previously described [13]. Cytokine and chemokines IL-2, IL-4, IL-5, IP-10, IFN-γ, and TNF-α were measured in the culture medium of PBMCs following SUDV and SUDV proteins GP, NP, VP30 and VP40 stimulation using Q-Plex technology (Quansys Biosciences, Logan, Utah, USA) according to the manufacturer's instructions. Data were collected with a Quansys Imager (Quansys Biosciences) and results analyzed using Q-View (Quansys Biosciences).

2.6. IgG ELISA

The levels of circulating anti-SUDV and anti-SUDV protein antibodies were determined by chemiluminescence ELISA, as previously described [22]. ELISA cut-off values for IgG-positive immunoreactivity were set as mean + 2*STDV above of negative control sera.

2.7. IgG subclass isotyping

Antigen-specific IgG subclass isotyping was performed as described elsewhere using an IgG isotyping kit (Southern Biotech, Al USA) according to the manufacturer's instructions [24]. Briefly, 96 well U shape plates were pre-coated with SUDV or SUDV proteins GP, NP, VP30 and VP40, were blocked with 10% skim milk and incubated with sera at a 1:50 dilution followed by a set of subclass-specific anti-human IgG 1–4 (Southern Biotech, Al USA) and anti-mouse HRP (Jackson immunodetect, PA USA). Infinite F200 pro-Elisa reader then acquired relative light units (RLU) results (Tecan, Austria) and converted to pg/ul concentrations.

2.8. Plaque reduction neutralization test

Plaque reduction neutralization assays (PRNT) were performed as previously described [13]. Neutralization was measured for 1:10 dilutions of sera. The positive neutralization cut off value was the mean + 2*STDV of control set of negative sera. Plaque reduction neutralization assays were performed in the BSL-4 lab of United States Army Medical Research Institute of Infectious Diseases USAMRIID (Fort Detrick, Frederick, MD, USA).

2.9. Antibody- FcγR depended reporter assays

The cell-based reporter system to quantitate pathogen-specific antibody binding to FcγRs receptors was described elsewhere [24]. Briefly, 96 well cell culture plates were pre-coated with SUDV, and SUDV proteins GP, NP, VP30 and VP40 at 2.5 μg/ml concentration diluted in 1xPBS and incubated for 2 h, followed by blocking solution with 1% BSA for 1 h. Human diluted samples sera diluted 1:200 were added and incubated for additional 1 h. BW-FcγR cells (Mouse BW5147 thymoma cells (ATCC TIB-47™) with cloned sequences encoding the human extracellular portion of FcγRs fused to murine CD3ζ chain (Invitrogen, CA USA)) $50^{*}10^{3}$ cells/well were supplemented and incubated in RPMI 10% (*v*/v) FCS medium for 16–18 h. Each step was followed by a primary wash step with 1xPBS at 200 μl/well volume. All incubations were at 37 °C in an atmosphere of 5% CO_2. Supernatants of activated cells were collected after 16–18 h incubation and analyzed for mIL-2 concentration using a commercial ELISA kit (Biolegend, CA USA).

2.10. Statistical analysis

Statistical analyses were performed using GraphPad Prism software, v6.01 (GraphPad Software, Inc. LA Jolla, CA, USA). The Spearman nonparametric test assessed the correlation. Differences in cytokine values between study groups were assessed by analysis of variants (ANOVA) and Wilcoxon rank sum test; p-values represent 2-sided p values, and p values <.05 were considered statistically significant. Principal Component Analysis (PCA) plots were generated in ClustVis web tool using Redge packages [27].

3. Results

3.1. Study population

Peripheral blood mononuclear cells (PBMCs) and match sera samples were collected 15-year post-infection from confirmed EVD survivors who lived in or around the Gulu district of Uganda during the initial outbreak in 2000–2001 and were hospitalized for between 10 and 25 days. There are 15 survivors, five male and ten females, with ages between 18 and 61 years (mean/median age: 40.7/45 y). Three additional samples of matched sera and PBMCs were collected from uninfected donors who were local community members. These were used as controls. All subjects were healthy and reported a lack of autoimmune diseases, cancer and past hospitalizations, unrelated to ebolavirus disease (EVD) (Table 1), suggesting a lack of confounding infections. Survivors reported uniform treatment (supportive care only) and symptoms during and after the acute illness (not shown). The characteristics for each SUDV survivor and control are shown in Table 1.

3.2. T cell immunity

3.2.1. Circulatory T cell immune responses in long-term SUDV survivors favour CD4+ T cell immunity

T cell responses in PBMC were assessed by measuring intracellular levels of the cytokines Interferon gamma (IFN-γ), Interleukin (IL) 2, and *Tumor necrosis factor alpha* (TNF-α) in CD4 + CD40L+ and CD8 + CD107a + T cells by flow cytometry with or without antigen-stimulation. The gated strategy is presented (Fig. S1). Levels of secreted cytokines IFN-γ, IL-2, IL-4, IL-5, Interferon gamma-induced protein 10 (IP-10) and TNF-α were also measured in supernatants of stimulated and non-stimulated PBMCs by multiplex ELISA.

Control stimulation antigens were first used to confirm the assay function. No cytokine response was observed in either survivor or

Table 1
Characteristics of SUDV survivors and health controls participants. Personal information of SUDV survivors' and healthy controls, including gender, age, health status and treatment medication is presented. The data was obtained during sample collection and from the donor clinical history during the SUDV outbreak. Sn denotes a survivor, Cn denotes a control.

Serial No	Gender	Collection data			Age at infection	Outbreak data	
		Age	Illness	Medications		Hospitalization time	Treatment during infection
S1	f	34			19	14 d	s.t.
S2	f	23			8	14 d	s.t.
S3	f	50			35	14 d	s.t.
S4	m	45			30	10 d	s.t.
S5	m	20			5	21d	s.t.
S6	f	61			46	21 d	s.t.
S7	f	57			42	21 d	s.t.
S8	f	57	None	None	42	25 d	s.t.
S9	m	48			33	14 d	s.t.
S10	f	20			5	14d	s.t.
S11	f	46			31	12 d	s.t.
S12	f	32			17	14d	s.t.
S13	m	47			32	14d	s.t
S14	f	41			26	21d	s.t
S15	m	32			17	14d	s.t
C1	f	39			–	–	–
C2	f	39	None	None	–	–	–
C3	f	28			–	–	–

F- female, m- male, s.t – supportive treatment.

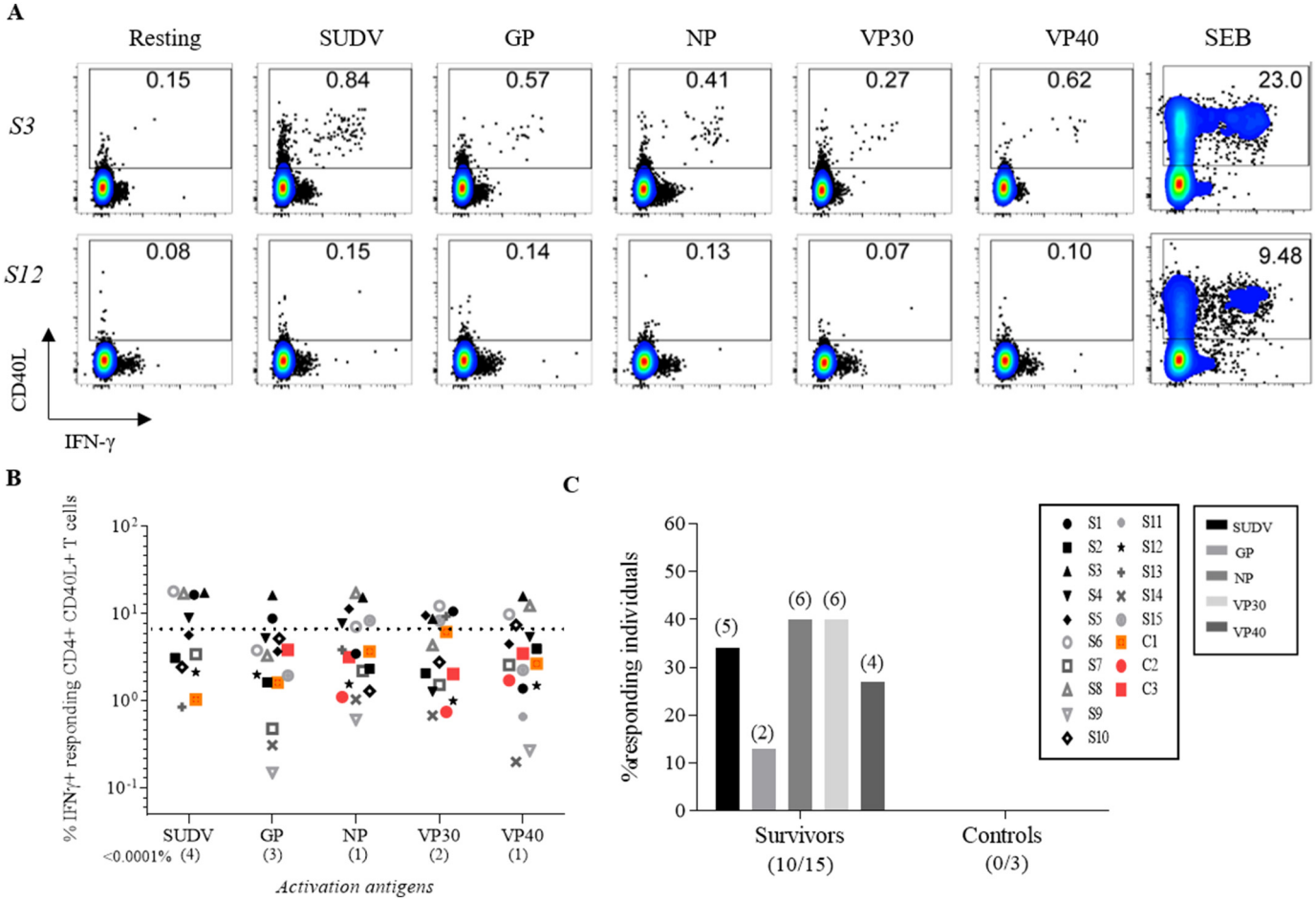

Fig. 1. Survivor CD4 + CD40L+ T cell response to SUDV antigen stimulation. PBMCs from SUDV survivors (n = 15) and uninfected controls (n = 3) were collected and stimulated with irradiated Sudan virus (SUDV) or one of the SUDV proteins: GP, NP, VP30, VP40. 2 h post stimulation, monensin (4 μg/mL) was added and the PBMCs incubated for a further 16 h. Activated CD4+ T cells and control resting cells from the same individuals were then assessed for intracellular levels of the cytokines IFNγ, IL-2 and TNF-alpha by flow cytometry. Representative cytometry data for IFNγ from two survivors are shown (A). Samples were acquired on an LSRII (BD Biosciences) and analyzed with FlowJo (vX, TreeStar). We defined a cell as IFNγ-responding ($IFN\gamma_+$) if it displayed a minimum of one log10 increase of mean fluorescence intensity over the average of all unstimulated (resting) PBMCs. For all patients, the fractions of CD4 + CD40L+ T cells that are $IFN\gamma_+$ are shown in (B). The cut-off value, showing the fraction of resting cells exhibiting a positive response, is indicated by a dashed line. A patient sample was judged $IFN\gamma_+$ if the fraction of CD4 + CD40L+ T cells exhibiting a positive response was above the cutoff. The bracketed values are the number of samples showing zero response (<0.000001%). Sn denotes a survivor, Cn denotes a control sample. The fractions of patient samples showing positive IFN-γ responses following antigen stimulation are shown in (C), categorized by activating antigen and by sample vs. control. Bracketed values at the column heads are the numbers of positive response samples for each antigen. The bracketed values at the column feet are the fraction of survivors in the group showing positive IFN-γ response following any antigen stimulation.

uninfected control CD4+ or CD8+ cells following stimulation with negative control antigens; irradiated MARV or MARV GP (Fig. S1), whereas stimulation with the positive control antigen, SEB, led to elevated levels of IFN-γ, IL-2, and TNF-α (Fig. S1). The corresponding measures of PBMC-secreted IFN-γ, IL-2, IL-4, IP-10, and TNF-α, measured by multiplex ELISA were consistent with the flow cytometry data except for TNF-α, which showed elevated levels in survivors and in uninfected controls after MARV GP stimulation (Fig. S2).

To characterize the cellular phenotype (i.e. effector, memory) of survivor T cells, we assessed survivor SUDV-specific CD4+ and CD8+ T cell immune responses. PBMCs were first stimulated with irradiated SUDV and the intracellular levels of IFN-γ, IL-2, and TNF-α were measured in CD4 + CD40L+ and CD8 + CD107a + cells. Cells were considered cytokine-responding (cytokine+) if the cytokine signal was more than one log10 greater than the average mean signal of unstimulated (resting) cells of both survivors and uninfected controls. CD4+ and CD8+ T cells were considered activated (effector) if they were CD40L + or CD107a+, respectively, and were IFN-γ + [25,26]. Representative IFN-γ flow cytometry results in two survivors, S3 and S12, gated on CD40L + CD4+ T cells are shown (Fig. 1A). 35% of SUDV survivors (5 out of 15) showed positive CD4 + CD40L+ IFN-γ response following SUDV stimulation (Fig. 1B and Fig. 1C). Uninfected control sera showed no positive cytokine response following stimulation. In contrast to the CD4+ T cell immune responses, CD8 + CD107a + T cells isolated from survivors or uninfected control PBMC exhibited no positive responses of IFN-γ, IL-2, or TNF-α following SUDV stimulation (Fig. 2 and data not shown).

3.2.2. SUDV survivors exhibit long-term, antigen-specific CD4+ T cell immune responses

To assay antigen-specific cellular immune responses in SUDV survivors, we measured IFN-γ expression in CD4 + CD40L+ and CD8 + CD107a + T cells following SUDV protein GP, NP, VP30 or VP40 stimulation using the same approach as above. We observed that the four SUDV proteins elicited CD4 + CD40L+ T cell IFN-γ -specific responses (Fig. 1A-C). Of the SUDV proteins, NP and VP30 most frequently elicited a response in the patients, 40% (6 out of 15), followed by VP40, 27% (4 out of 15), and GP, 13% (2 out of 15) (Fig. 1B and Fig. 1C). No antigen elicited a response in uninfected control PBMC.

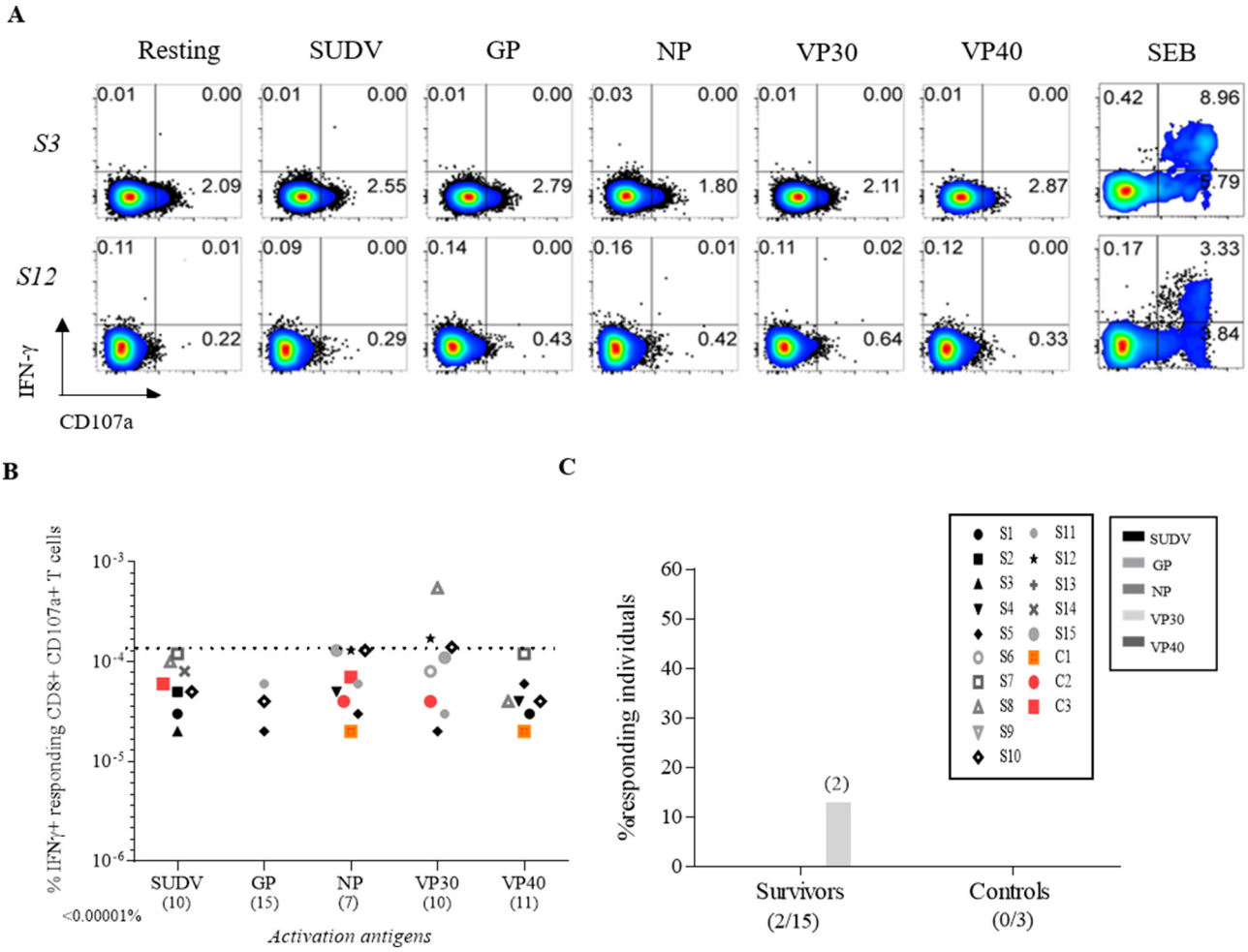

Fig. 2. Survivor CD8 + CD107a + T cell response to SUDV antigen stimulation. PBMCs from SUDV survivors (n = 15) and uninfected controls (n = 3) were collected and stimulated with irradiated Sudan virus (SUDV) or one of the SUDV proteins: GP, NP, VP30, VP40. 2 h post stimulation, monensin (4 μg/mL) was added and the PBMCs incubated for a further 16 h. Activated CD8+ T cells and control resting cells from the same individuals were then assessed for intracellular levels of the cytokines IFNγ, IL-2 and TNF-alpha by flow cytometry. Representative cytometry data for IFNγ from two survivors are shown (A). Samples were acquired on an LSRII (BD Biosciences) and analyzed with FlowJo (vX, TreeStar). We defined a cell as IFNγ-responding ($IFN\gamma_+$) if it displayed a minimum of one log10 increase of mean fluorescence intensity over the average of all unstimulated (resting) PBMCs. For all patients, the fractions of CD8 + CD107a + T cells that are $IFN\gamma_+$ are shown in (B). The cut-off value, showing the fraction of resting cells exhibiting a positive response, is indicated by a dashed line. A patient sample was judged $IFN\gamma_+$ if the fraction of CD8 + CD107a + T cells exhibiting a positive response was above the cutoff. The bracketed values are the number of samples showing zero response (<0.000001%). Sn denotes a survivor, Cn denotes a control sample. The fractions of patient samples showing positive IFN-γ responses following antigen stimulation are shown in (C), categorized by activating antigen and by sample vs. control. Bracketed values at the column heads are the numbers of positive response samples for each antigen. The bracketed values at the column feet are the fraction of survivors in the group showing positive IFN-γ response following any antigen stimulation.

In addition to IFN-γ, the fractions of CD4 + CD40L+ T cells responding to antigen stimulation by only IL-2 or TNF-α production were also measured in PBMC of SUDV survivors using the same protocol as for IFN-γ. The cumulative data are like antigen-provoked IFN-γ production. NP stimulation most frequently elicited IL-2 and TNF-α cytokine responses, followed by VP30 and VP40 (data not shown).

In contrast to these CD4+ data, SUDV proteins elicited negligible or no measurable cytokine response in CD8 + CD107a + T cells from survivors or controls (data not shown and representative IFN-γ flow cytometry results Fig. 2). Of the SUDV proteins, only VP30 evoked a positive response, albeit very low, in 13% of survivors (2 out of 15, Fig. 2B and Fig. 2C). No antigen elicited a response in uninfected controls. Since CD8+ T cell responses in SUDV survivors were practically absent, we focus our further analysis on survivors CD4+ T cell responses.

SUDV survivors exhibit antigen-specific CD4 + CD40L+ T memory cell response

For long-term CD4+ T cell-mediated immunologic memory, a subset of CD4+ T cells is required to differentiate into memory T cells [28]. One hallmark of CD4+ memory T cells is multiple cytokine production, particularly triple production, in response to antigen stimulation [28,29]. We, therefore, identified CD4+ T cells expressing combinations of IFN-γ, TNF-α, and IL-2 in response to antigen stimulation.

All seven possible combinations of cytokine-positive expression were observed in survivor CD4 + CD40L+ T cells following SUDV or SUDV proteins stimulation (Fig. 3A). As expected, no combination of cytokine-positive expression was observed in uninfected control CD4 + T cells (Fig. 3C and D and data not shown). The different combinations of all survivors' responses were aggregated into triple-cytokine positive (TP), double-cytokine positive (DP) and single-cytokine positive (SP) and the relative proportions of each in survivor CD4 + CD40L+ T cells calculated (Fig. 3B). More TP cells were observed than any other combination following stimulation with any of the antigens (Fig. 3A). Although other DP and SP responses were observed among the survivors, they were weaker and varied to a much greater extent among survivors; statistical analysis of cytokine responses of CD4 + CD40L+ stimulated cells vs unstimulated cells (resting) showed that only the elevated TP response to SUDV antigen stimulation was significantly higher (Fig. 3A). This significant TP response was observed for all the viral antigens. Irradiated SUDV complete antigen provoked the most robust response (47%), followed by NP, (34%), VP40 (30%), VP30 and GP (26% each) (Fig. 3A and Fig. 3B). Among the SUDV survivors, 40% (6 out of 15) exhibited a TP response following irradiated SUDV

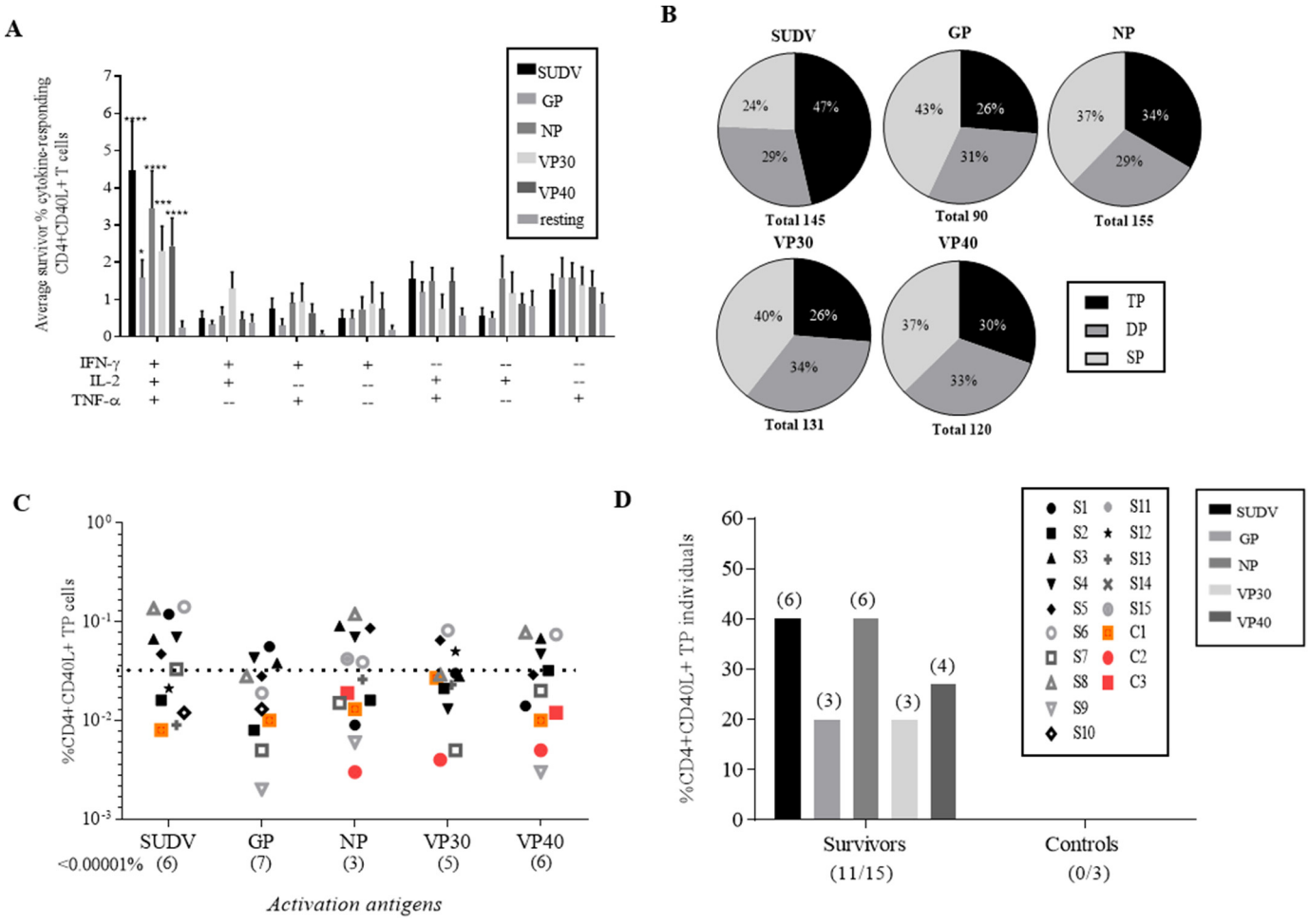

Fig. 3. Immunophenotyping of CD4 + CD40L+ T cell responses to SUDV antigen stimulation. The CD4 + CD40L+ T cell response data following antigen stimulation (Fig. 1) were used to assess combinatorial cytokine responses (A). Cytokine combinations are indicated at the bottom. Values are Mean ± SEM. Significance was established by analysis of variance (ANOVA), comparing responses to the corresponding values from the total resting cell population. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. The combinations of cytokines were aggregated into categories of single, double, and triple cytokine-responding cells (SP, DP, TP) and the mean fractions of cytokine-responding cells in each category presented separately for each indicated antigen (B). The fractions of CD4 + CD40L+ T cells from each patient that are TP are shown for each activating antigen (C). The bracketed values are the number of samples showing zero response (<0.0001%). Sn denotes a survivor, Cn denotes a control sample. The fraction of patient samples showing triple positive responses following antigen stimulation are shown in (D), categorized by activating antigen and by sample vs. control. Bracketed values at the column heads are the numbers of positive response samples for each antigen. The bracketed values at the column feet are the fraction of samples in the group showing positive cytokine response following any antigen stimulation.

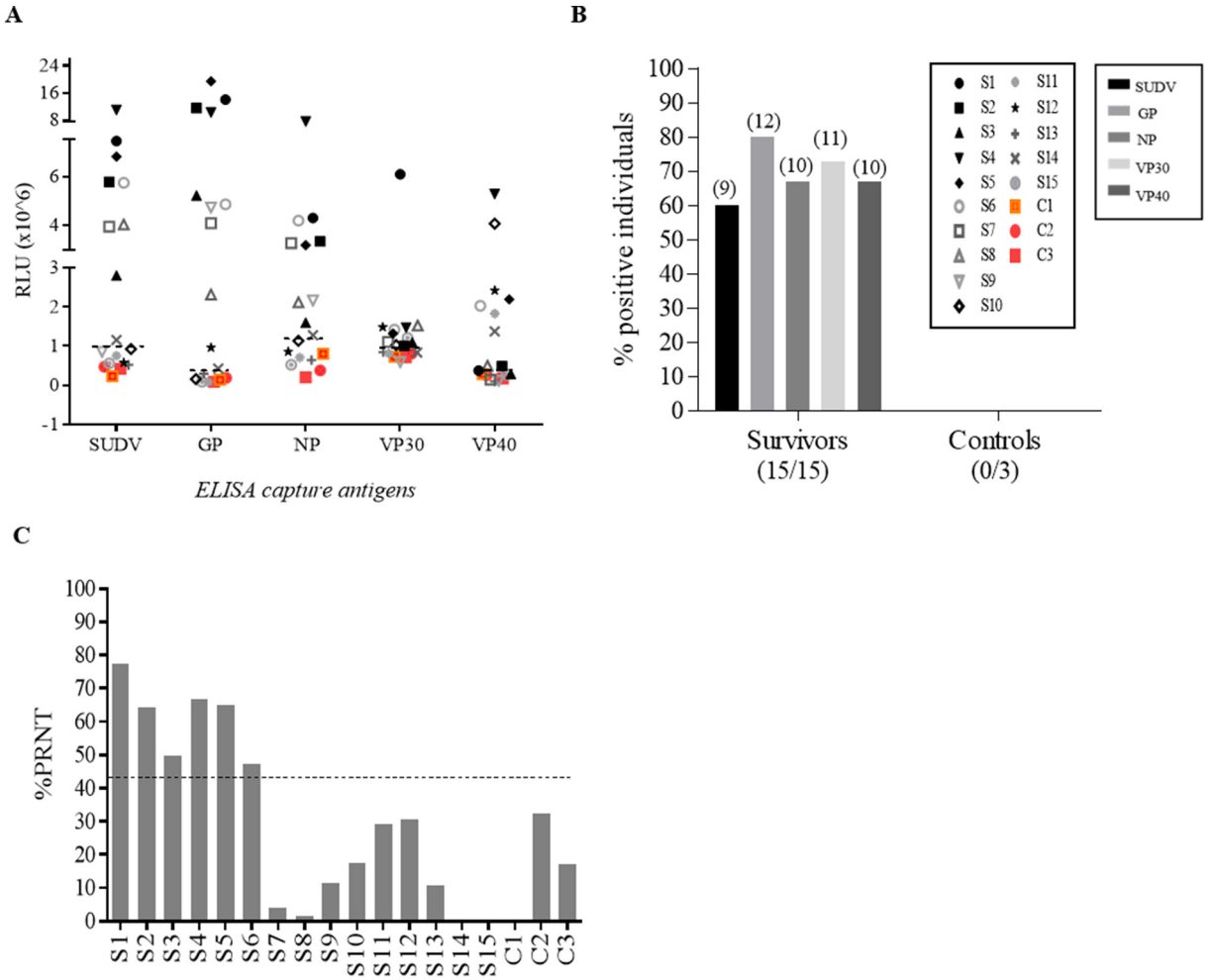

Fig. 4. SUDV survivor anti-SUDV IgG antibody response. Sera from SUDV survivors (n = 15) and uninfected controls (n = 3) were collected and analyzed for IgG immunoreactivity by ELISA (A and B). Irradiated Sudan virus (SUDV) or SUDV proteins: GP, NP, VP30, and VP40 were bound to assay plates, incubated with diluted sera and bound serum IgG was detected using an anti–human IgG-HRP antibody and Luminol/oxidizer HRP substrate. Sera samples were analyzed in triplicates, and mean values are reported as relative light units (RLU). Immunoreactivity was judged positive if the RLU was >2*STDV above the average for the control sera - indicated by a dashed line. Sn denotes a survivor, Cn denotes a control sample. The fractions of patient samples showing positive antigen-specific IgG response are shown in (B), categorized by capture antigen and by survivor vs. control. Bracketed values at the column heads are the numbers of positive response samples for each antigen. The bracketed values at the column feet are the fraction of samples in the group showing positive IgG response to any capture antigen. In vitro neutralization capacity was assessed by Plaque Reduction Neutralization Test (PRNT) on tenfold dilutions of patient sera (C). The sera were scored positive if the value was 2*STDV above the average value for the control sera (indicated by the dashed line).

stimulation, and NP stimulation, 27% after VP40 (4 out of 15), and 20% (3 out of 15) after VP30 and GP.

3.2.3. SUDV survivor CD4 + T cell response is Th1-skewed

To differentiate between Th responses, we stimulated donor PBMCs with SUDV antigen and then measured the levels of 6 diagnostic cytokines, INF-γ, IL-2, IL-4, IL-5, Interferon gamma-induced protein 10 (IP-10), and TNF-α, in the culture supernatants by multiplex ELISA. We defined positive responses following SUDV antigen stimulation based on the same criteria as described above for flow cytometry. We observed a wider distribution of positive antigen-specific cytokine responses (Fig. S3). Of the SUDV antigens used, irradiated SUDV induced the highest IFN-γ, IL-2, and IP-10 cytokine responses. As was observed by flow cytometry, NP stimulation triggered the highest IFN-γ and TNF-α responses. However, NP-induced positive TNF-α responses were also observed in uninfected controls. Overall, cytokine markers indicative of a Th1 response (IFN-γ, IL-2, TNF-α, IP-10) were elevated following SUDV antigen stimulation, whereas markers typical of a Th2 response, IL-4 and IL-5, were nearly absent in all survivors and uninfected controls, regardless of the antigen used to stimulate the PBMC (Fig. S3).

3.3. Anti-viral antibody response

3.3.1. SUDV survivors maintain virus-specific IgG antibodies

Evidence from naturally recovered Ebola survivors and recent clinical vaccine studies have demonstrated the importance of antibodies in successfully fighting a primary infection and their protective role in preventing secondary infection. We, therefore, characterized the humoral anti-SUDV IgG component in survivor sera. Matched serum samples were collected from the 15 SUDV survivors and the three uninfected controls. Anti SUDV IgG was measured by ELISA against irradiated SUDV antigen. Sera showing a value higher than 2*STDV above the average for the control sera were scored as positive. Our results showed that 60% of survivors (9 of 15) maintained IgG virus-specific antibodies to irradiated SUDV (Fig. 4A and Fig. 4B, Table S2). As expected, uninfected controls showed no IgG positive response.

3.3.2. SUDV survivors exhibit robust antigen-specific anti-viral antibody responses

To determine the antigen-specificity of the observed humoral response, we repeated the IgG response measures using the four SUDV proteins GP, NP, VP30 and VP40 instead of irradiated SUDV antigen

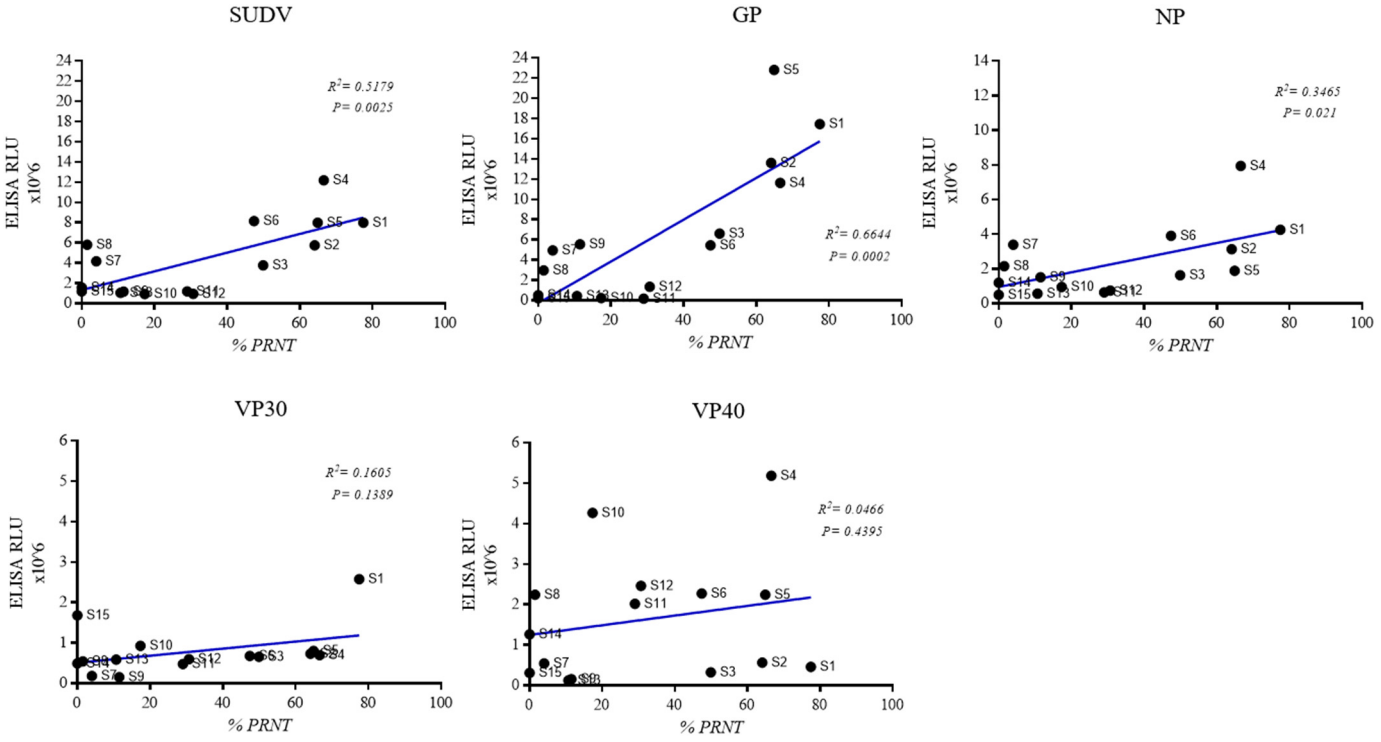

Fig. 5. Correlation analysis between antigen-specific IgG ELISA and PRNT assays of sera from SUDV survivors. Correlation between survivor sera immunoreactivity to the various SUDV antigens by ELISA and in vitro SUDV neutralization were performed (D For each correlation individual results, linear regression, including R^2 and P value, are presented. P values <0.05 were considered statistically significant.

and the same criteria for scoring sera positive. SUDV survivors exhibited robust IgG responses against all SUDV viral proteins tested (Fig. 4A and Fig. 4B, and Table S2). All SUDV survivors demonstrated IgG immunorecognition of at least one of the SUDV antigens (Fig. 4B). 80% of SUDV survivors (12 out of 15) were IgG positive to GP, 73% (11 out of 15) to VP30, and 67% (10 out of 15) to NP and VP40 (Fig. 4A and Fig. 4B). Since the molecular properties of IgG antibody potentially affect their function, the subclass composition of IgG to SUDV and SUDV proteins was determined by isotyping. We found IgG1 to be the most prominent subclass antibody, regardless of SUDV target antigen used (Fig. S4). IgG3 and IgG4 subclass antibodies were also detected in survivors but, the levels were much lower and were mainly against viral proteins NP and VP40 (Fig. S4).

3.3.3. SUDV survivors are capable of mediating in vitro neutralization and antibody Fc-dependent response

While antibodies have been suggested to protect against Ebola virus, the mechanism is unclear. To address the potential function of the observed SUDV-specific serum antibodies, we first used a plaque reduction/neutralization test (PRNT) to determine whether survivor serum could neutralize live virus in vitro. A serum sample that neutralized SUDV plaque formation (PRNT) with the value above the mean + 2STDV of uninfected controls was scored as neutralizing. Six of the fifteen survivors (S1-S6) had neutralizing sera (Fig. 4C). This neutralization correlated with the observed IgG immunoreactivity to SUDV (P-value = .0025), and the SUDV proteins GP (P-value = .0002) and NP (P-value = .021), Fig. 5. As expected, no sera of uninfected controls were neutralizing (data not shown).

Since only half of SUDV survivors had serological neutralization capacity and given recent data suggesting a role for non-neutralizing antibodies in viral protection, we also studied a second IgG-mediated protective mechanism of action, antibody Fc-dependent response via FcγRI and FcγRIIIA receptors. Cell culture plates were pre-coated individually with SUDV antigens and incubated with survivor's sera, and then BW-FcγR-expressing cells. Antigen-specific IgG survivor sample was scored as positive for FcγR activation if the IL-2 production of BW-FcγR cells was higher than 2*STDVs above the average of uninfected controls. IgG antibodies directed against SUDV protein GP had the most potent capacity to mediate an FcγRI response (Fig. 6A and Fig. 6C). 87% of SUDV survivors (13 out of 15) mediated antibody Fc-dependent response via FcγRI by antibodies directed to GP, 54% (8 out of 15) to irradiated SUDV antigen, 40% (6 out of 15) to NP and VP40, and 20% (3 out of 15) to VP30. (Fig. 6A and Fig. 6C). Antibody Fc-dependent activation through the FcγRIII receptor was also detected in survivors, although responses were lower in magnitude and mainly mediated by antibodies directed against SUDV VP40 or irradiated SUDV (47%, 7 out of 15) (Fig. 6B). In total, 87% of SUDV survivor sera (13 out of 15) mediated antibody Fc-dependent responses via FcγRI or FcγRIIIA (Fig. 6C). Analysis between antibody Fc-dependent responses and neutralization capacity showed significant responses via FcγRI receptor by antibodies directed against irradiated SUDV, and SUDV proteins GP, NP and VP40 independently of neutralization capacity (Fig. 6D). A significant correlation between FcγRI response to SUDV GP (P-value = .0089) or FcγRIII response to irradiated SUDV (P-value 0.0237) and in vitro neutralization was also observed (Fig. 7).

3.4. Profiles of memory immunity in SUDV survivors

To understand the interplay of the immune responses seen in SUDV survivors, we also performed a correlation analysis between the cellular and humoral findings across the multiple immune assays. The results (summarized in Table S1) revealed in general positive correlation between cellular and humoral responses in SUDV survivors. A significant and robust correlation between CD4+ T cell effector and memory responses was seen following stimulation with any of the SUDV antigens. There was also a significant and robust correlation between IgG antibody FcγRI-mediated response following GP stimulation and neutralization. The complete correlation results between the various immune assays following each SUDV antigen stimulation are presented in Table S1.

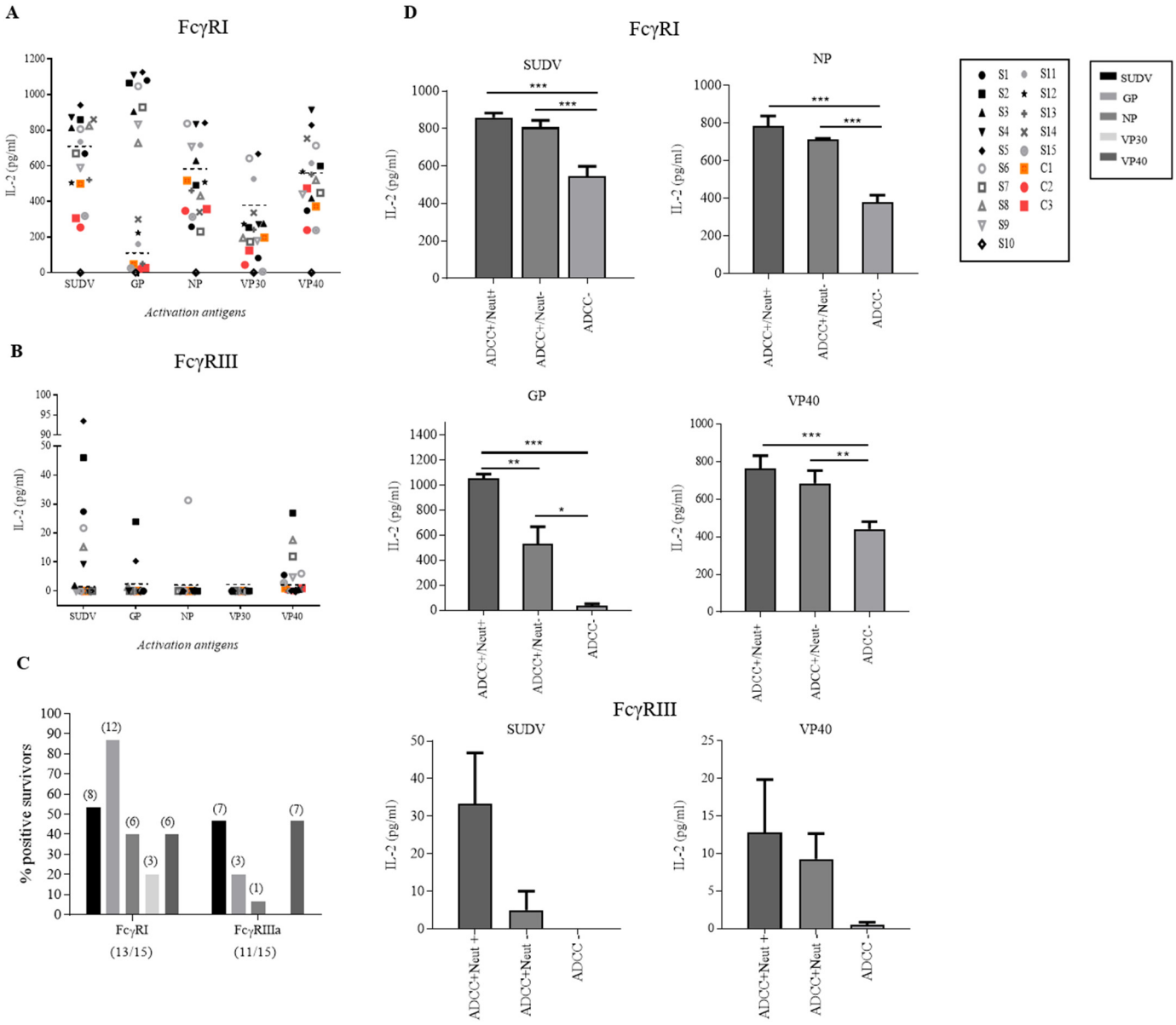

Fig. 6. SUDV survivor antibody FcγRs depended response. Irradiated Sudan virus (SUDV) or SUDV proteins: GP, NP, VP30, and VP40 were bound to assay plates, incubated with diluted patient sera and then the plates washed. FcγRI-expressing (A) or FcγRIIIa-expressing (B) BW cells were then added and the plates incubated for 16–18 h. Supernatants were then assayed for IL-2 levels by ELISA. Sera leading to an IL-2 level >2*STDVs above the average of uninfected controls were considered positive (cut-offs indicated by dashed lines). Sn denotes a survivor, Cn denotes a control sample. The fractions of survivor sera provoking a positive antibody-dependent FcγR response are shown in (C), categorized by capture antigen and by FcγR receptor. Bracketed values at the column heads are the numbers of positive response sera for each antigen. The bracketed values at the column feet are the fraction of sera in the group provoking a positive antibody-dependent FcγR response to any capture antigen. The survivor sera-provoked IL-2 levels were then categorized by antigen (SUDV, GP, NP, VP40), Fcγ receptor type (FcγRI, FcγRIIIa), antibody FcγRs depended positive or negative (Ab-FcγR+, Ab-FcγR-), and neutralizing capacity assayed in Fig. 4 (Neut+, Neut-) (D). Values are Mean ± SEM. Significance was established by Analysis of variance (ANOVA) comparing responses. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The small number of survivors with FcγRI positive responses of VP30 antibodies and FcγRIIIa positive responses of GP, NP and VP30 antibodies did not permit analysis.

To complement the correlation analysis, we also examined the cellular and humoral immune responses in each survivor (Table S2) to identify specific profiles of immunity in SUDV cohort of survivors. The data suggest two groups of long-recovered survivors exhibiting different memory immunity profiles. One consists of survivors with both cellular and humoral responses (cluster 1 (c1), survivors 1, 3, 4, 5, 6, and 8) and other showing only a partial or a complete lack of measurable responses (cluster 2 (c2), survivors 2, 7, and 9–15).

To determine if these groups are substantially different from each other, we performed a comparative analysis of the immune responses between them and healthy controls using Principal Component Analysis (PCA). Six immune responses of SUDV survivors: CD4+ T cell effector, TP cytokine secreted T memory, IgG immunoreactivity, neutralization and antibody FcRs (I and III)-dependent responses, were analyzed. The PCA and Heatmap analyses (Fig. 8) discriminated between the two subgroups of SUDV survivors (c1, marked in red and c2, marked in blue) following stimulation with SUDV, viral protein GP, or viral protein NP. PCA analysis also showed that the c2 subgroup exhibited immune responses similar to those of the healthy control group (c3 marked in green).

4. Discussion

To better understand the components and processes driving the development of persistent memory immunity following *Filoviruses* infection, we evaluated for the first time SUDV antigen-specific cellular and

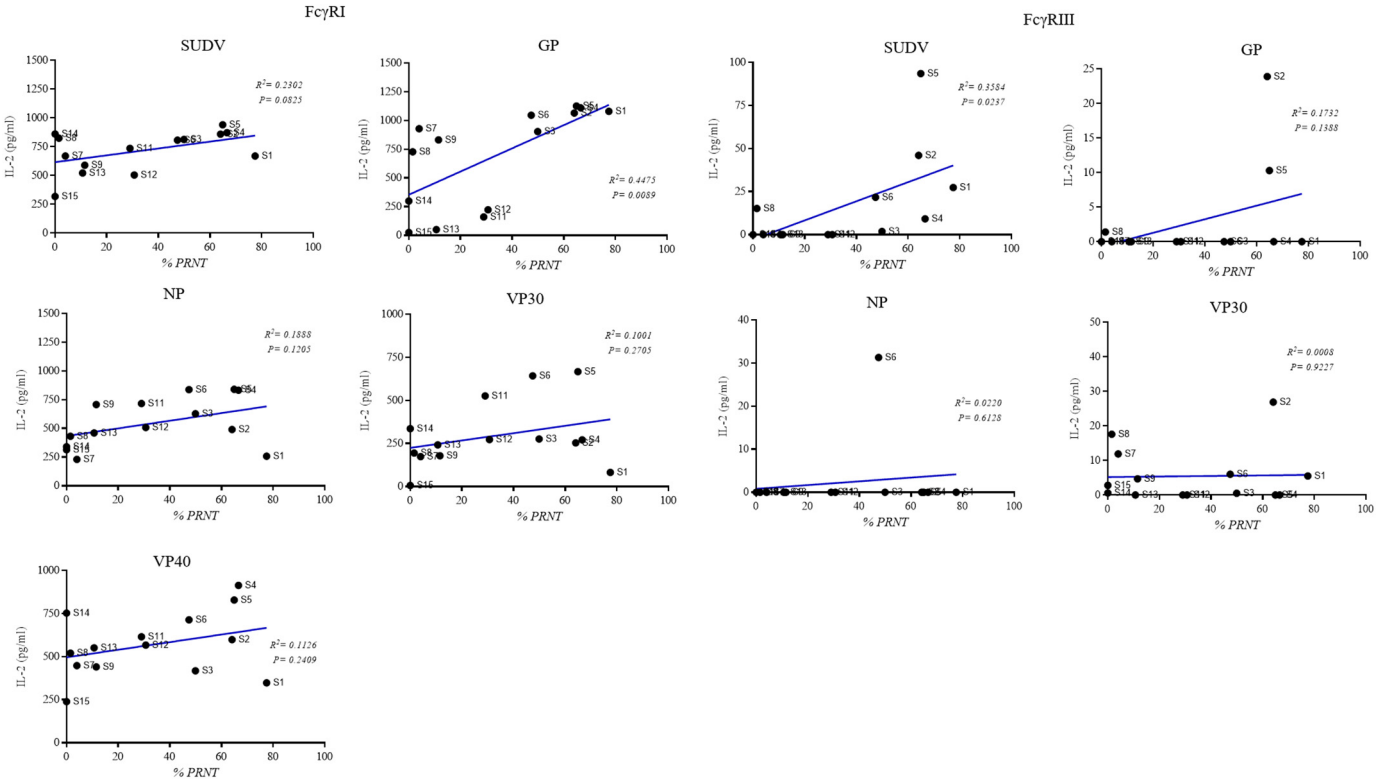

Fig. 7. Correlation analysis between FcγRs dependent responses and PRNT assays of sera from SUDV survivors. Correlations between the antibody-dependent FcγR responses mediated by SUDV antigens and in vitro SUDV neutralization were determined by Spearman correlation analysis. For each SUDV antigen the correlation results, including individual data, linear regression, R^2 and P value, are presented. P values <0.05 were considered statistically significant.

humoral immune compartments of recovered SUDV survivors 15 years post infection. The study included 15 individual survivors' representing the approximately 30-member SUDV-gulu cohort. Our results showed that 15 years following infection, some SUDV survivors maintained robust CD4+ T effector cell responses, especially to viral proteins NP and VP30 (Fig. 1) but exhibited minimal activated CD8 + CD107a + T cell response to any SUDV antigen tested (Fig. 2). Most responding CD4+ T cells exhibited triple-positive cytokine responses, suggesting that they were memory cells. This response was provoked most strongly by viral protein NP (Fig. 3).

Coupled to cellular immunity, survivors also maintained strong antigen-specific IgG responses to all tested SUDV proteins (Fig. 4). These were mostly of the IgG1 subclass. Approximately 85% of SUDV survivors had functional IgG antibodies in their sera with capacity for either in vitro SUDV neutralization or antibody Fc-dependent response. (Fig. 4 and Fig. 6). Neutralization correlated with ELISA IgG

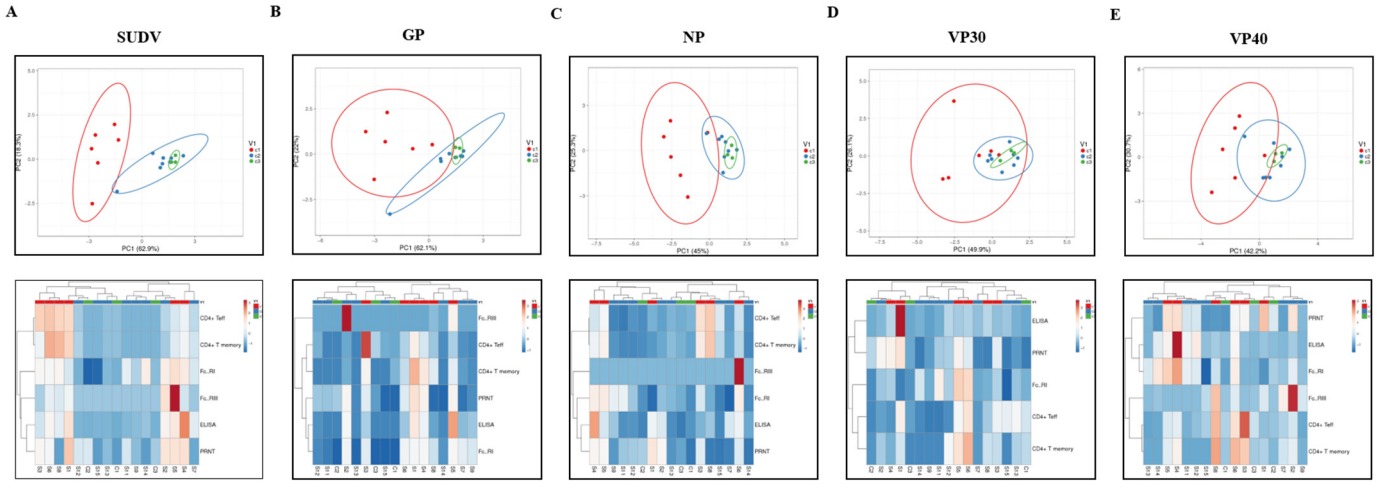

Fig. 8. Clustered analysis in SUDV survivors and controls. SUDV survivors were split into two subgroups. Subgroup 1 (c1) consisted of survivors who had demonstrated both cellular and humoral immunity (S1, S3, S4, S5, S6, and S8), and subgroup 2 (c2) consisted of survivors who showed partial or lack of either cellular or humoral immunity (S2, S7, S9, and S11-S15). A cluster analysis in these two subgroups of survivors and control individuals (C1-3) was done using Principal Component Analysis (PCA) and CD4+ T cell effector, TP cytokine secreted T memory, IgG ELISA immunoreactivity, PRNT and antibody FcγRs (I and III)-dependent response assay values. PCA plot and Heatmaps following antigen-specific stimulation are shown for SUDV (A), GP (B), NP (C), VP30 (D), and VP40 (E). SUDV survivor's subgroup c1 is indicated in red, subgroup c2 indicated in blue, and the healthy controls c3 are indicated in green. S10 was excluded from this analysis since FcRs (I and III)-dependent responses were initially not tested in this donor.

immunoreactivity to SUDV viral proteins GP and NP (Fig. 5). The antibody Fc-dependent responses via FcγRI and FcγRIIIA receptors were mainly by IgGs against GP and VP40. The antibody Fc-dependent response through FcγRI receptor was independent of sera neutralization capacity (Fig. 6).

Overall, naturally recovered SUDV survivors-maintained antigen-specific memory CD4+ T cell responses and functional IgG antibodies for decades following infection. These responses were mediated by specific viral proteins. Analysis of CD4+ T cell responses showed that survivors exhibiting responses to the complete virus also respond to individual protein stimulation. For a few survivors a positive response was only observed to individual proteins and not the complete virus. This could be due to higher protein concentrations when stimulating with individual proteins or to differences in protein conformation affecting epitope presentation.

The CD4+ T cell responses detected in SUDV survivors are particularly relevant if, as has been suggested, they provide a "superior" response [29] and, as has been observed in other viral infections, are more likely to mediate long-term protection [28,30–32]. Why this cellular response persists is unclear; however, several possible explanations can be proposed, including high viral load during infection, re-exposure or "bio-mimetic" antigens, or persistence of the virus in immune-privileged sites in the body, as was recently observed in West Africa survivors [33,34].

The observation that the SUDV NP protein was a prime target of the persistent cellular immune response consists with data from other studies in EVD survivors during acute infection and the early phases of convalescence, which also identified EBOV NP as the primary target of CD8 + T cell responses [6,7,9].

Studies of patients and survivors of the West Africa EBOV outbreak indicated that activated CD8+ T cells are present during acute infection and the early convalescence stage [6–9,11]. In the long-term survivors we studied, there was minimal CD8 + CD107a + T cell response following SUDV antigen stimulation. Since studies of this same cohort of survivors during the acute stage of infection demonstrated CD8+ cellular responses [35], it appears that long-term memory immunity of SUDV survivors heavily favours the CD4+ T cell compartment. This has been observed in other cohorts of filovirus survivors [10] and other viral infections, suggesting that the CD8+ T cell response significantly diminishes over time [36]. Alternately, the lack of detectable CD8+ T cell immune responses in SUDV survivors could be due to small cell population, the time point of collection (15 years post infection), or the use of whole antigens (rather than peptides) for stimulation.

The antibody response data in the SUDV survivors revealed broad and robust antigen-specific IgG responses years following infection. These responses consist with our previous reports from this cohort of survivors [13,23,37]. IgG antibodies of these survivors were primarily IgG1 subclass, results which were in line with recently published data from this survivor's cohort but using only GP as antigen target [24]. Our study further indicated that elevated levels of IgG3 and IgG4 antibodies, particularly against viral proteins NP and VP40. These IgG antibody isotypes may suggest a link between IgG isotype and functionality, such as a non-GP neutralization pathway, as recently suggested by others [38]. Our observation of sustained neutralization capacity in this SUDV cohort of survivors consist with our previous report, done in 10 years post infected survivors, and further extended our knowledge regarding the duration of this antibody-mediated response [13]. Although the magnitude of this neutralization capacity seems to wane over time. More, we found SUDV survivors were capable of mediating antibody Fc-dependent responses, particularly by antibodies directed against SUDV and SUDV viral proteins GP and VP40. The antibody Fc-dependent response showed prominent binding to FcγRI, as compared to FcγRIIIA, results consistent with a recent report from this group of SUDV survivors [24]. The difference in binding affinity could be due to antibody-Fc modifications such as antibody subclass and type-dependent changes in glycosylation patterns, as previously suggested in other viral infections [39]. It is unclear why antibodies from SUDV survivors favour an FcγRI mediated response. It could be that this adaptive combined innate immunity (via macrophages) may contribute to the containment of viral replication and allow time for T cell responses to develop. Overall, the antiviral IgG results demonstrate several pathways of antibody-mediated function in recovered survivors. This agrees with recent reports from other EBOV survivors [40].

The alarming frequency and magnitude of Ebola virus outbreaks in recent years across Africa has led to accelerated development of vaccine candidates [16,17]. An effective vaccine would be capable of inducing long-term and broad responses [15,16] from both humoral and cellular immune compartments; these qualities are essential for lasting adequate protection from Ebola [3,12]. To date, all advanced candidate vaccines have been based solely on a single Ebola virus protein, GP. Despite being a prime target of the immune response, it is still unclear if this single viral protein may be enough since we do not yet understand the relative roles of other viral proteins in establishing long-term memory immunity.

In this respect, our study is particularly relevant. We observed multiple immune responses in individual SUDV survivors 15 years after infection. Analysis of those responses revealed that the memory immunity composition in long-recovered survivors consists of both humoral and cellular memory immune responses. More, a significant correlation was observed between those responses. However, this persistent immune memory response was not present in all survivors, and a subset of the cohort was found to have a partial or complete lack of measurable immune memory immunity. These findings are consistent with our previous studies [13] and other observations that noted differences in subtypes of Ebola virus survivors depending on different viral-immunological features and carrier states [41]. Comparing the subsets of SUDV survivors did not indicate any significant differences with respect to survivors age at time of infection, gender, hospitalization period or treatment (Table 1, Table S3 and data not shown).

Although the profile of immunity in SUDV survivors may consist of immune responses developed due to viral misdirection, it is more likely that the combination of these multiple immune pathways would provide an essential advantage against recurrent viral infection. Our data suggest that GP, although a principal target of an immediate response, fails to provoke a long-term cellular response. Such long-term cellular memory response was developed against other viral proteins, specifically NP and VP40. It may be beneficial to include these in future vaccines. The breadth and length of antigen-specific CD4+ cellular responses and the lack of CD8+ T cell response in SUDV long term survivors also suggest that vaccine's ability to trigger a CD4+ response may be an equally important indicator of its potential than its ability to trigger a CD8+ response. Collectively the data presented in this study improves our understanding of the immunological landscape of Ebola virus memory immunity and should benefit the development of future vaccines.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2019.07.021.

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Author contributions

Experiments were designed by AS, SWS, JJL, ASH, JMD, VY and LL. AS, SWS, ASH, JJL, acquired and processed blood samples. SUDV recombinant viral proteins were constructed, expressed, and purified by SG

and AS. SWS and ASH performed flow cytometry experiments. Secreted cytokines analysis and serological assays were done by AS. AIK performed the PRNT assays. OR preformed ADCC assays. IgG isotyping was performed by AE and SFM. Data analysis was performed by AS, SWS, AP, JMD, CD, VY and LL. The manuscript was written by AS, CD, SWS and edited by AP, ASH, JMD, and LL.

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In memory of Dr. Leslie Lobel - a great scientist, mentor, and beloved friend who succumbed to cancer while finalizing this work. He will be greatly missed.

Declaration of competing interest

The authors declare no competing commercial interests. Opinions, interpretations, conclusions and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army.

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