

Epitopes of Naturally Acquired and Vaccine-Induced Anti-Ebola Virus Glycoprotein Antibodies in Single Amino Acid Resolution

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The Ebola virus (EBOV) can cause severe infections in humans, leading to a fatal outcome in a high percentage of cases. Neutralizing antibodies against the EBOV surface glycoprotein (GP) can prevent infections, demonstrating a straightforward way for an efficient vaccination strategy. Meanwhile, many different anti-EBOV antibodies have been identified, whereas the exact binding epitopes are often unknown. Here, the analysis of serum samples from an EBOV vaccine trial with the recombinant vesicular stomatitis virus-Zaire ebolavirus (rVSV-ZEBOV) and an Ebola virus disease survivor, using high-density peptide arrays, is presented. In this proof-of-principle study, distinct IgG and IgM antibodies binding to different epitopes of EBOV GP is detected: By mapping the whole GP as overlapping peptide fragments, new epitopes and confirmed epitopes from the literature are found. Furthermore, the highly selective binding epitope of a neutralizing monoclonal anti-EBOV GP antibody could be validated. This shows that peptide arrays can be a valuable tool to study the humoral immune response to vaccines in patients and to support Ebola vaccine development.

1. Introduction

The Ebola virus (EBOV) is, due to its high fatality rate of 50% on average, one of the most threatening pathogens in our society.^[1] The 2014–2016 outbreak of EBOV in West Africa was the largest since the virus discovery and the outbreak in the Democratic Republic of Congo is still ongoing since 2018 with the current overall case fatality ratio of 67%.^[2] Both are caused by the Zaire ebolavirus species.^[1] Thus, several passive and active immunotherapies against EBOV infection are currently under development. Few monoclonal antibodies (mAbs) for passive immunization have been shown to alleviate the Ebola virus disease (EVD).^[3–8] Active immunotherapy has some advantages over passive. The currently most promising approach in EVD prevention is the recombinant vesicular stomatitis

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virus-Zaire ebolavirus (rVSV-ZEBOV) vaccine, based on the rVSV, carrying the EBOV glycoprotein (GP).^[1] The rVSV-ZEBOV recently received conditional market authorization and, thereby, represents the world's first licensed Ebola virus vaccine. Operational research during the current Democratic Republic of Congo outbreak revealed 97.5% efficacy for the prevention of EVD.^[9] The surface protein of EBOV, the GP, is the target of neutralizing antibodies. It is cleaved by the enzyme furin into two subunits, GP1 and GP2. The cleavage products GP1 and GP2 are connected through a disulfide linkage between Cys53 and Cys609 and are inserted in the viral membrane as a trimeric complex, which mediates the entry into host cells.^[10–13] GP1 contains receptor-binding regions, the glycan cap, and the mucin-like domain.^[11] After the uptake of the virus by the cell, cathepsins remove the glycan cap and the mucin-like domain, leading to the exposure of the receptor-binding region.^[14–16] GP2 serves as the membrane fusion subunit and contains the internal fusion loop, two heptad repeats, and the transmembrane domain.^[11] In addition to the surface bound GP, there are two other gene products of the GP gene, resulting from stuttering of the polymerase at the editing site of GP: the secreted (soluble) glycoprotein (sGP) and the small secreted glycoprotein. With $\approx 70\%$, sGP is the main gene product.^[17] It is highly secreted from infected cells and detected in the serum of EBOV-infected hosts.^[18] The secreted form shares the first 295 residues with the surface GP,^[12] which therefore may decoy antibodies and help the virus to escape the immune response.^[19–22]

To develop new vaccines, it is important to identify those epitopes, which are targeted by protective antibodies in humans. For this purpose, peptide microarrays^[23] can be used to rapidly study antibody interactions (e.g., mAbs or serum samples) with a large number of linear epitopes, which can be screened by simple incubation and fluorescence scan analysis. Advances in technology development, which for example rely on the patterning of co-polymers for the synthesis of biomolecules,^[24] make the rapid production of peptide microarrays feasible.^[25–27] High-density peptide microarrays have been used for the development of diagnostic biomarkers, for example, in Malaria, Zika virus, and Chagas disease,^[28–30] as well as in the analysis of therapeutic antibodies against *C. difficile*.^[31] Additionally, epitopes recognized by antibodies elicited after vaccination against infectious diseases, such as Tuberculosis, Malaria, and Tetanus, can be identified.^[32–34]

Here, we report the use of high-density peptide arrays to exactly map the epitopes of naturally acquired antibodies against EBOV GP from volunteers, vaccinated with rVSV-ZEBOV, and one survivor of EVD. Our results show that many distinct epitopes are targeted by the humoral immune response. Generally,

Table 1. Patient and vaccine recipient information.

Sample	Vaccine dose [pfu] ^{a)}	Day of serum collection after vaccination	ELISA OD ^{b)} (450–630 nm)	Arbitrary ELISA units ^{b)}
1	2×10^7	28	0.811	4156
2	3×10^5	28	0.826	3874
3	3×10^5	56	1.220	6233
4	3×10^5	56	0.928	4483
5	2×10^7	56	0.428	1703
6	3×10^6	180	0.446	1424
7	2×10^7	180	0.802	3190
Survivor ^{c)}	n.a.	n.a.	3.565	22 737

^{a)} plaque forming units (pfu); ^{b)} ELISA data, representing the total sample-specific EBOV-binding antibodies (IgG) in optical density (OD) and normalized arbitrary ELISA units (AEU); ^{c)} Sample was collected 2 months later than the survivor serum sample, which was analyzed with the peptide array.

we could identify many distinct epitopes in the EBOV GP, with significant differences as well as overlaps in EBOV vaccine recipients in comparison to EVD infection.

2. Results

2.1. Enzyme-Linked Immunosorbent Assay to Detect EBOV-Specific Antibodies

We analyzed the sera from seven vaccines with rVSV-ZEBOV expressing EBOV GP, one EVD survivor from 2014,^[35] and one neutralizing monoclonal antibody (mAb) 3T0331.^[36] The sera from volunteers, once vaccinated with rVSV-ZEBOV, were collected on days 28 ($n = 2$), 56 ($n = 3$), and 180 ($n = 2$) after vaccination. Each sample is from a different vaccine recipient, at different time points after vaccination, using different vaccine doses (Table 1).

First, we performed an Enzyme-linked immunosorbent assay (ELISA) to test the samples for EBOV-specific antibodies.^[37] Microtiter plates were coated with inactivated whole EBOV particles. Thus, in this assay, all antibodies against EBOV proteins were detected, resulting in a generally much stronger signal in the survivor sample (as shown in ^[37]), since the survivor has developed antibodies against several EBOV proteins (GP, NP, VP40). Therefore, a direct comparison of survivor and vaccines is not possible. Nevertheless, the investigated serum samples of vaccines were all positive for anti-GP antibodies.

2.2. Epitope Mapping Using Peptide Microarrays

Then, we mapped the amino acid (AA) sequence of the EBOV GP (676 AA, National Center for Biotechnology Information [NCBI] accession number AAG40168.1) as 662 overlapping 15-mer peptide fragments with a lateral shift of one AA (14 AA overlap). With this information, we obtained nine high-density peptide microarrays (PEPperPRINT GmbH, Germany), displaying these 662 peptides as spot duplicates.

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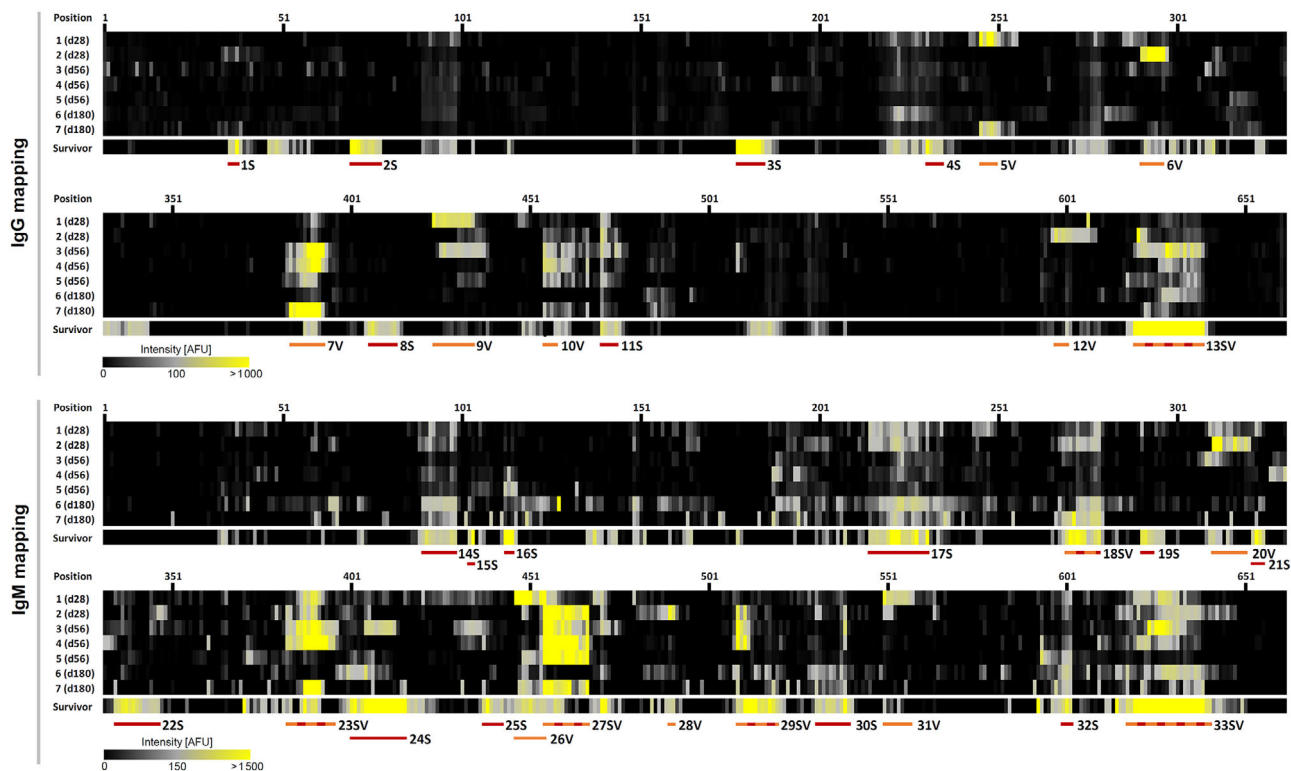


Figure 1. Peptide microarray data shown as a heat map (top: IgG response, bottom: IgM response). The EBOV GP (676 AA) was mapped as 662 spots of 15-mer peptides with a lateral shift of one AA. Sera from seven rVSV-ZEBOV-GP vaccines (numbered) and one EVD survivor were analyzed on separate microarrays. The vaccines are listed with the day of serum collection after vaccination. The defined epitopes are named by their origin from vaccines (V, in orange), survivor (S, in red) or both (SV, in orange and red). The identified peptide epitopes are summarized in Table 2.

Next, we performed the peptide microarray experiments. The peptide arrays were incubated with the eight diluted sera or the respective mAb, followed by incubation with fluorescently labeled secondary antibodies against human IgG and IgM (see Experimental Section). The obtained fluorescence scans were analyzed regarding the intensity of the peptides and visualized as a heat map (Figure 1). The IgG response shows a more specific binding pattern (less noise) than the IgM response, which reflects the higher specificity of IgGs. A binding epitope is defined by at least two neighboring peptide binders (one AA offset in the EBOV-GP) above certain intensity thresholds. For the IgG response, we defined the initial threshold as 500 arbitrary fluorescence units (AFU). The end of the epitope is defined, when the intensity of neighboring peptide(s) is below 250 AFU. For the IgM response, the initial intensity threshold for an epitope is 750 AFU and 375 AFU for the neighboring peptide(s). The identified peptide epitopes are summarized in Table 2.

Some of the epitopes in the IgG and IgM response show significant overlap (Figure 1). Furthermore, some epitopes are IgM specific, but do not induce a strong binding for IgG, especially in the day 28 patient samples. Comparing the antibody responses from the vaccines, we see differential responses. As we would expect, we observe the tendency that IgG epitopes show higher intensities in the sera of days 56 and 180 after vaccination, whereas in the earlier serum samples (day 28 and 56), the IgM response is higher. We did not observe a vaccine dose dependent (pfu) antibody response, but more samples should be analyzed to validate

this. However, we observed a qualitative correlation between the arbitrary ELISA units of the vaccine recipients and the arbitrary fluorescence intensity of the epitopes. For example, vaccine recipients 5 and 6 show the lowest signals in ELISA, as well as a low number of epitopes with lower fluorescence intensity.

The number of epitopes found in the vaccines sera within the N-terminal half of the EBOV-GP is low and not homogeneous. Some epitopes can be found in the IgG and IgM response of the survivor, with almost no overlap to the vaccines samples. Yet, for the following C-terminal half, several epitopes (IgG: 13SV; IgM: 23SV, 27SV, 29SV, 33SV) are recognized by antibodies of the survivor and the vaccines. Our strict selection criterion might have caused us to miss some shared epitopes (e.g., 11S), where weaker signals are present in both, the survivor and the vaccines.

The IgG epitope mapping of the potentially neutralizing mAb 3T0331 indicated one position within the EBOV GP at position 491–506 with the AA sequence GLITGRRRTREAIVN.^[36]

Furthermore, we compared the epitopes found by us with published GP epitopes from EVD survivors, which were analyzed with ELISA (Becquart et al.), and from vaccinated individuals (prime/boost vaccination with chimpanzee adenovirus 3 encoding EBOV GP (ChAD3) and modified vaccinia virus Ankara encoding the GP of different filoviruses and the nucleoprotein of Tai forest ebolavirus), analyzed with yeast surface peptide-display assay (Rijal et al.). The epitopes are illustrated within the domains of the GP protein sequence (Figure 2A).^[38,39] Additionally, we

Table 2. Amino acid position and sequence of the GP epitopes of IgG and IgM aligned from top to bottom according to GP amino acid position (see Figure 1).

IgG			IgM		
Epitope	Position	Sequence	Epitope	Position	Sequence
1S ^{a)}	36–52	GVIHNSTLQVSDVDKLV	14S	90–113	SGVPPKVVNYEAGEWAENCYNLEI
2S	70–92	LEGNQVATDVPSATKRWGFRRSGV	15S	103–118	EWAENCYNLEIKKPDG
3S	178–199	EGVVAFLILPQAKKDFSSHPL	16S	113–129	IKKPDGSECLPAAPDGI
4S	231–249	EYLFVNDNLTYVQLESRFT	17S	215–245	STTIRYQATGFGTNETEYLFVNDNLTYVQLE
5V ^{b)}	246–265	SRFTPQFLLQLNETIYTSG	18SV	270–293	TGKLIWKVNPEIDTTIGEWAFWET
6V	291–312	WETKKNLTKIRSEELSFTVV	19S	291–308	WETKKNLTKIRSEELSF
			20V	311–334	VSNGAKNISGQSPARTSSDPGTNT
			21S	322–339	SPARTSSDPGTNTTTEHDH
			22S	335–361	TTEDHKIMASENSAMVQVHSQGREAA
7V	384–407	PDNSTHNTVPYKLDISEATQVEQH	23SV	383–410	GPDNSTHNTVPYKLDISEATQVEQHRRR
8S	406–427	QHRRRTDNDSTASDTPSATTAA	24S	400–433	EATQVEQHRRRTDNDSTASDTPSATTAAAGPPKAE
9V	424–449	TTAAGPPKAENTNTSKSTDFLDPAT	25S	438–457	SKSTDFLDPATTPSQNHSE
10V	455–473	HSETAGNNNTHHQDTGEE	26V	447–469	ATTTSPQNHSETAGNNNTHHQDT
11S	471–489	EESASSGKGLITNTIACV	27SV	455–481	HSETAGNNNTHHQDTGEESASSGKGL
			28V	490–505	AGLITGRRRTRREIV
			29SV	509–534	PKCNPNLHYWTTQDEGAAIGLAWIPY
			30S	531–554	WIPYFGPAEAGIYIEGLMHNQDGL
			31V	550–571	NQDGLICGLRQLANETTQALQL
12V	598–616	GGTCHILGPDCCIEPHDW	32S	600–616	TCHILGPDCCIEPHDWT
13SV ^{c)}	620–653	TDKIDQIHFDFDKTLPDQGDNDNWWTGWRQWIP	33SV	618–655	NITDKIDQIHFDFDKTLPDQGDNDNWWTGWRQWIPAG

^{a)}The defined epitopes, named by their origin from survivor (S); ^{b)}vaccine recipients (V); ^{c)}or both (SV).

mapped and highlighted our found IgG epitopes on the 3D structure of the GP (Figure 2B–D).

Compared to the seven epitopes (1A–7A) from Becquart et al.,^[38] five epitopes coincide with our defined IgG epitopes from the survivor and vaccines. In fact, four of these (1A, 3A, 6A, 7A) overlap with the antibody response of our survivor sample (1S, 4S, 8S, 11S). Rijal et al.,^[39] defined three rather large epitopes (1B–3B), of various lengths. The first (1B) overlaps with one epitope of the survivor (3S), the second (2B) includes one survivor epitope (4S) and one epitope of a vaccine (5V), and the third (3B) has a small overlay of 2 amino acids with one vaccine epitope (6V).

3. Discussion

Peptide microarrays are a useful high-throughput platform for epitope mapping. With our approach, we identified the epitopes of the acquired IgG and IgM response in parallel against EBOV GP from rVSV-ZEBOV vaccine recipients and one survivor of EVD. For the first time, we used overlapping peptides to map the EBOV GP with an offset of one amino acid to determine the exact position and length of the epitopes. Furthermore, for the first time, we followed the development of isotype switch in rVSV-ZEBOV vaccine recipients at different time points after vaccination with this approach, which will help to investigate vaccination boost strategies.

The mAb 3T0331, which is a potently neutralizing anti-GP antibody, binds to a peptide on the microarray at the position AA491–506 in the GP, which correlates well with the determined binding site from Ehrhardt et al.^[36] They have determined a binding of the N-terminal GP2 and parts of GP1 with main interactions to Val505 and Glu502, but not to Gln508. Thus, peptide microarrays can be readily used to identify the antigen binding sites of mAbs or antibodies found in more complex samples like sera with high precision.

Recently, the Viral Hemorrhagic Fever Immunotherapeutic Consortium screened a global collection of 168 monoclonal antibodies against EBOV GP.^[40] Although being a landmark contribution to the EBOV field, the workflow is highly labor-intensive: To determine the binding domain of the mAbs within the GP, they first performed ELISA to detect antibody binding to different forms of the GP antigen, followed by imaging the complexation of the mAb Fab fragments with GP using electron microscopy. For in depth evaluation of the bound epitope, they performed alanine scanning mutagenesis to create point mutated antigens and characterize the essential epitope residues for mAb binding. They could cluster the mAbs into the respective epitope classes of GP base, glycan cap, fusion loop, GP1 head, GP1/2, HR2, mucin-like domain, and unknown binding. Similarly, Rijal et al.,^[39] isolated 82 antibodies from plasmablasts or B cells from 11 vaccines with ChAD3 EBOV after day 7 and 28. A cocktail of four of these antibodies targeting the glycan cap, the receptor binding region, and the base, was protective in guinea pigs when given at day 3 after EBOV infection. They performed

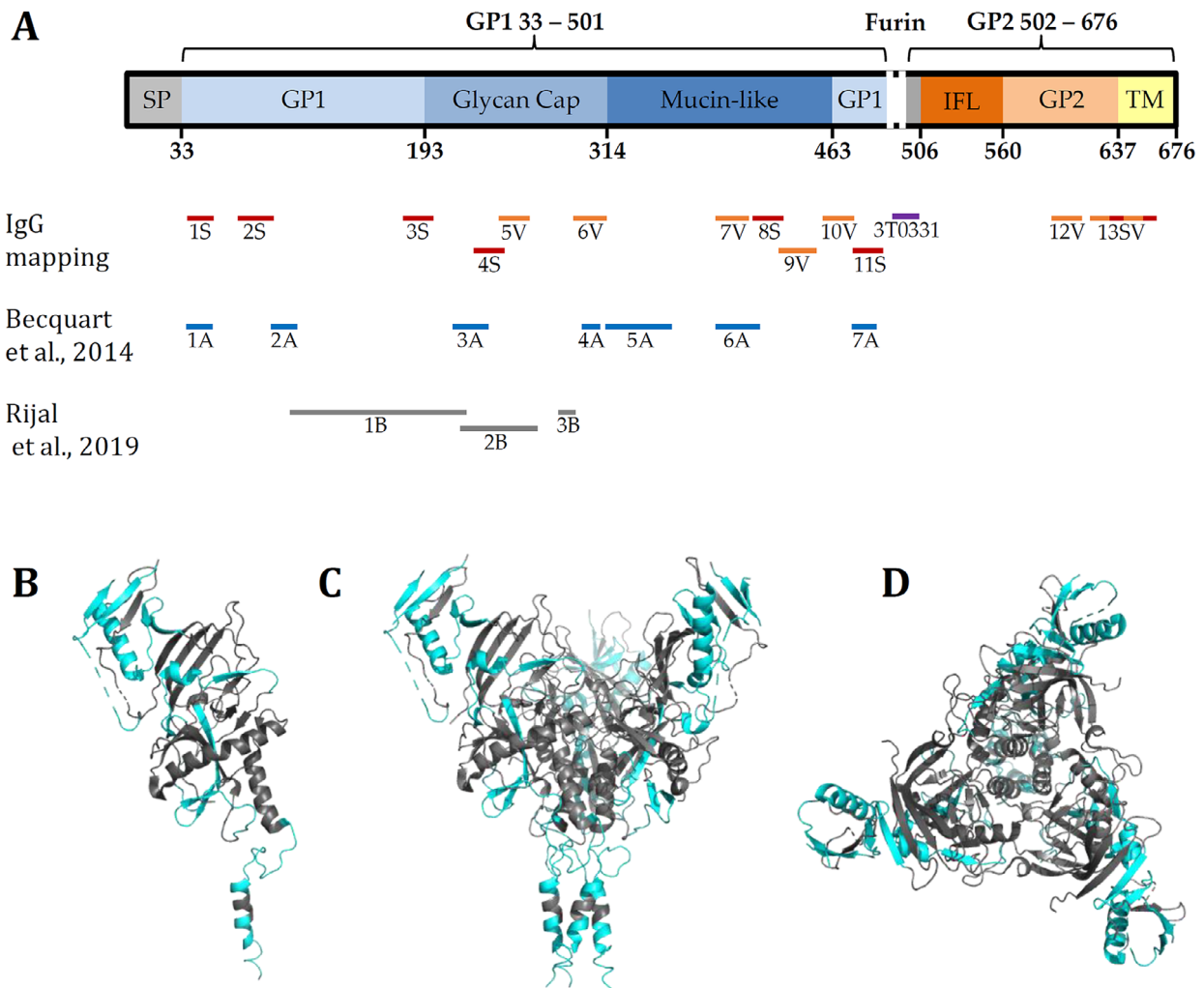


Figure 2. Epitopes of the EBOV GP. A) Comparison of the immunogenic epitopes of the EBOV GP. The herein presented IgG epitopes (S/V) compared to epitopes published in Becquart et al.,^[38] (A) and Rijal et al.,^[39] (B); for details, also see Tables S2 and S3, Supporting Information. Structural domains of the envelope glycoprotein (GP) from Saphire et al.:^[40] SP: signal peptide; mucin-like: mucin-like domain; IFL: internal fusion loop, TM: transmembrane domain. 3D view of the (incomplete) EBOV GP structure with the herein identified IgG epitopes (vaccines and survivor) highlighted in cyan (generated with PyMOL from Protein Data Bank^[41] file 5jq3^[42]—Crystal structure of Ebola glycoprotein). B) Side view of GP monomer C) side view of GP trimer D) Top view of GP trimer. For more details on missing sequences, see Table S4, Supporting Information .

a yeast surface peptide-display assay to identify the GP epitopes bound by these mAbs. The advantage of this display technology is the possibility to identify longer and conformational epitopes, but it lacks precise amino acid resolution. P. Becquart et al.,^[38] identified linear B cell epitopes of GP by ELISA of synthetic 15-mer peptides with an overlap of 11 amino acids. First, they screened two pools of different sera from EVD survivors and identified 19 peptides, which were recognized by IgG. Then, they used these peptides for further epitope mapping of 21 survivor sera. The drawback of this approach is the lower resolution of only 11 amino acids overlap, which might cause false negative results. In addition, pooling of sera makes it impossible to distinguish individual antibody responses.

Thus, peptide microarrays can offer an attractive and rapid alternative to the afore mentioned cost- and labor-intensive methods and still provide high-resolution epitope mapping. This

screening platform has advantages, such as reduced laboratory efforts due to parallel screenings, minimal demand of sample volumes and its ability to be used for diverse samples. For in-depth analysis of essential amino acids within the epitope sequences, amino acid substitution analysis can be performed.^[43] This may help to elucidate the crucial amino acids for vaccine development. To investigate a possible dose dependent response, more samples and different time points need to be analyzed. The applied peptide arrays also have their limitations, since they contain exclusively linear peptides and cannot identify antibodies that bind conformational or discontinuous epitopes.

We show that peptide arrays can be a versatile tool for highly precise epitope identification of a large number of mAbs or different antibody classes in sera of patients or vaccine recipients. Therefore, peptide arrays are useful in vaccine development

to reduce laboratory efforts and minimize demand of sample volumes.

4. Experimental Section

rVSV-ZEBOV Vaccines: Serum samples of rVSV-ZEBOV vaccinated volunteers were obtained from an open-label, dose-escalation phase 1 trial performed in Hamburg (ClinicalTrials.gov; NCT02283099). Volunteers were vaccinated once with rVSV-ZEBOV-GP with a dose of 3×10^5 , 3×10^6 , or 2×10^7 pfu. Sera were collected on day 28, 56, 180 after vaccination.^[44,45] The protocol was approved by the Institutional Review Board of the University Medical Center Hamburg-Eppendorf, Germany.

ELISA Analysis: ELISA was performed as described by Krähling et al.^[37] Briefly, microtiter plates coated with EBOV or mock antigen, were washed three times with PBST (0.1% (v/v) Tween 20 in phosphate-buffered saline) and then blocked with PBS containing 5% milk powder. Washing procedure was repeated three times with PBST. Human sera/plasma and controls were diluted 1:200 in PBST containing 1% milk powder and allowed to react with the antigens. After washing plates three times with PBST, polyclonal HRP-coupled antibodies and TMB substrate and stop solution were used for detection. The optical density (OD) was determined at 450–630 nm using an automated spectrophotometer. Each control and serum were analyzed in duplicate, and mean OD value of each sample on mock antigen is subtracted from OD value on EBOV antigen to obtain corrected OD values. To calculate arbitrary ELISA units (AEU), the straight line equation of the standard curve on each plate is determined by linear regression analysis. Positive samples had an AEU of 1000 and higher, and negative samples were set to 500 AEU.

Generation of Peptide Microarrays: For the epitope mapping of patient sera with peptide microarrays, the Ebola virus glycoprotein, obtained from NCBI (AAG40168.1, 676 amino acids, <https://www.ncbi.nlm.nih.gov/protein/11761750>) was used. The GP sequence was cut in silico into 662 overlapping 15-mer peptide fragments, with an overlap of 14 amino acids between two neighboring peptides. These sequences were ordered as custom peptide arrays from PEPperPRINT GmbH (Heidelberg, Germany). Each microarray contained five copies of the Ebola GP array displayed as 15-mer peptide duplicate spots (2×662 individual spots per array).

Incubation and Analysis of Peptide Microarrays: Before incubation of the serum samples, the arrays were pre-swollen for 15 min with 300 μ L PBST (0.05% [v/v]) at room temperature and orbital shaking with 150 rpm. To avoid unspecific binding of the serum antibodies, the arrays were blocked with blocking buffer (MB-070, Rockland Immunochemicals Inc., Limerick, USA) for 30 min, 150 rpm, room temperature. After short washing with PBST, 200 μ L of the mAb 3T0031 diluted to 0.01 mg mL⁻¹ and sera diluted 1:500 in staining buffer (10% [v/v] blocking buffer in PBST) were incubated overnight, 150 rpm, at 4 °C. To remove unbound serum components, the arrays were shortly washed three times with PBST. The human serum antibodies were detected with fluorescently labeled secondary antibodies: 0.5 mg mL⁻¹ Anti-Human IgG-Fc Fragment cross-adsorbed DyLight 680 conjugated (A80-304D6, Bethyl Laboratories, Montgomery, USA), 1.0 mg mL⁻¹ Human IgM (mu chain) Antibody DyLight 800 conjugated (609-145-007, Rockland Immunochemicals Inc., Limerick, USA) and 1.0 mg mL⁻¹ anti-HA-peptide antibody (RT028, Bio X Cell, New Hampshire, USA) labeled with Lightning-Link Rapid DyLight 680 (327-0010, Innova Biosciences Ltd., Cambridge, United Kingdom). Therefore, the secondary antibodies were diluted 1:2000 in staining buffer and applied to the microarrays for 30 min, 150 rpm, room temperature. To remove unbound secondary antibodies, the arrays were shortly washed three times with PBST. Finally, the arrays were dipped in 1 mM Tris HCl pH 7.4 and dried in a jet of air. To monitor unspecific binding of the secondary antibodies (Anti-Human IgG 680, Anti-human IgM 800), the arrays were pre-stained and scanned before incubation with the samples using the same method. The arrays were scanned and fluorescence signals were detected at 700 nm and 800 nm with an Odyssey Scanner (LI-COR Biotechnology Inc., Lincoln, Nebraska, USA). Analysis of the scans was performed using

PepSlide Analyzer software (SICASYS Software GmbH, Heidelberg, Germany).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

F.F.L. is named on a patent for the production of microarrays. All other authors declare no conflict of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

Author Contributions

J.H. and V.K. contributed equally to this work. J.H. developed the peptide array content and performed all microarray related experiments and data analysis. V.K. performed the ELISA experiments. C.D., V.K., S.B., and M.M.A. performed and supervised all vaccine trial related experiments, T.W. collected the survivor sample and F.K. provided the monoclonal antibody 3T0031. F.F.L. supervised the microarray experiments and analysis. S.B., M.A., and F.F.L. supervised the project. All authors wrote and revised the manuscript.

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Ebola virus, epitope mapping, human sera, neutralizing antibodies, recombinant vesicular stomatitis virus-Zaire ebolavirus

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