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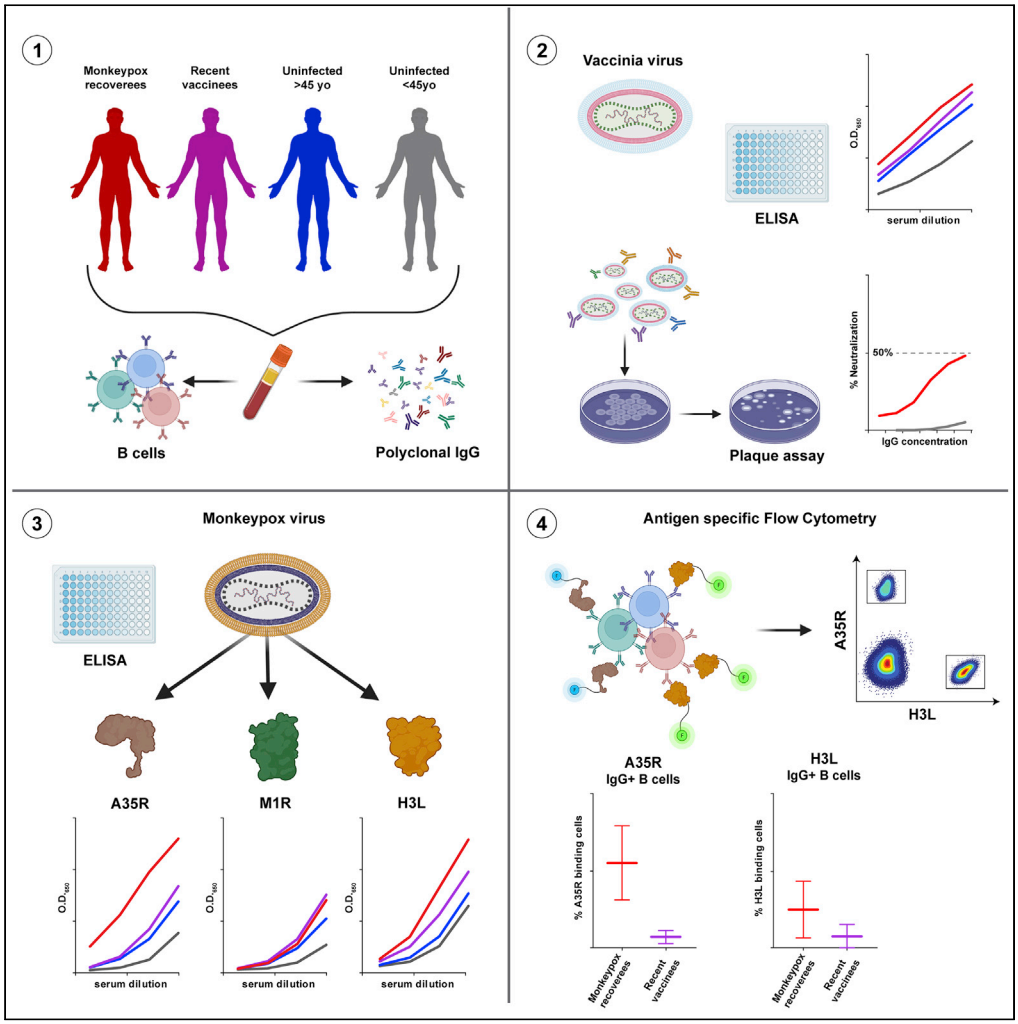

Ron Yefet, Nadav Friedel, Hadas Tamir, ..., Eli Sprecher, Tomer Israely, Natalia T. Freund

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Highlights

MPXV recoverees develop antibodies that target MPXV antigens A35R and H3L

Antibody response to A35R is higher in MPXV recoverees than in vaccinees

A35R and H3L-specific IgG+ B cell are more common in MPXV recoverees than vaccinees

Recent and past VACV vaccinated donors display similar antibody response to VACV

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Monkeypox infection elicits strong antibody and B cell response against A35R and H3L antigens

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SUMMARY

Monkeypox virus (MPXV) resides in two forms; mature and enveloped, and depending on it, distinct proteins are displayed on the viral surface. Here, we expressed two MPXV antigens from the mature, and one from the enveloped form, and tested their reactivity to sera of 11 MPXV recoverees while comparing to sera from recently and past vaccinated individuals. 8 out of 11 recoverees exhibited detectable neutralization levels against Vaccinia Lister. Sera from all recoverees bound strongly to A35R and H3L antigens. Moreover, the responses to A35R were significantly higher within the recoverees compared to both recently and past vaccinated donors. Lastly, A35R- and H3L-specific IgG$^+$ B cells ranging from 0.03-0.46% and 0.11–0.36%, respectively, were detected in all recoverees (A35R), and in 9 out of 11 recoverees (H3L). Therefore, A35R and H3L represent MPXV immune targets and could be used in a heat-inactivated serological ELISA for the identification of recent MPXV infection.

INTRODUCTION

Monkeypox virus (MPXV) is a member of the Orthopoxvirus genus and is responsible for Monkeypox disease.[1–3] The recent MPXV outbreak is the largest recorded outbreak in non-endemic countries to date.[4] As of December 28, 2022, over 82,000 people outside Africa were infected in 103 non-endemic countries, with the majority of cases detected in Europe and the Americas.[5,6] The infection results in blisters, fever and discomfort, with case mortality rate currently nearing 0.1% (for the 2022 outbreak).[6] Diagnosis of MPXV infection is primarily based on polymerase chain reaction (PCR) to detect the MPXV nucleic acids.[7] However, as infection rates continue to increase there is a need for rapid antigen-based serological assays that can be performed at non-BSL3 point of care sites. Moreover, serological assays can promote the understanding of both T cell and B cell responses and lead to the isolation of neutralizing antibodies that can be later examined as therapeutics. Lastly, serological assays can highlight potential targets for vaccine candidates.[8–11]

MPXV expresses approximately 25 membrane proteins on the mature virion (MV), a form that is dominant during inter-host transmission, and additional 6 proteins on the enveloped virion (EV), a form that is dominant during intra-host transmission.[12,13] Studies conducted on the related Vaccinia virus (VACV) show that entry to host cells is mediated through interactions with glycosaminoglycans, and through fusion with plasma membrane at neutral pH (mostly EV), or through low pH-expedited endocytosis (mostly MV).[14,15] Furthermore, several VACV proteins have been identified as important for viral attachment and entry,[14,15] some of whom were found to be targeted by antibodies elicited in immunized mice, infected macaques and both infected and vaccinated humans.[16–18] However, the main serological and B cell markers accompanying MPXV infection in humans are still not characterized. In the current report we examined the reactivity of three MPXV antigens to MPXV convalescent sera and compared it to the responses elicited by Vaccinia virus-based vaccine.

RESULTS

Sera from MPXV recoverees bind VACV and exhibit some VACV neutralization

To investigate the antibodies elicited following MPXV natural infection, we recruited a cohort of 11 MPXV recoverees 33–62 days post infection. All recoverees were diagnosed in Israel between May and June 2022 (Table 1). All the donors were males between the ages of 23 and 39, who were PCR-confirmed to be infected with MPXV, and exhibited a variety of symptoms including rash, disease characteristic blisters

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Table 1. MPXV recoverees' clinical data

Patient ID	Age	Sex	PCR confirmation	Rash	Fever	Number of blisters	Time from infection to sample collection (days)[a]
Mpx01	30	Male	Yes	Yes	Yes	10–20	38
Mpx02	33	Male	Yes	Yes	Yes	<10	33
Mpx03	39	Male	Yes	Yes	Yes	>20	62
Mpx04	37	Male	Yes	Yes	Yes	10–20	48
Mpx05	39	Male	Yes	Yes	Yes	10–20	47
Mpx06	34	Male	Yes	Yes	No	<10	60
Mpx07	26	Male	Yes	Yes	No	10–20	47
Mpx08	36	Male	Yes	Yes	Yes	10–20	NA
Mpx09	23	Male	Yes	Yes	Yes	10–20	51
Mpx10	24	Male	Yes	Yes	Yes	10–20	51
Mpx11	32	Male	Yes	Yes	No	10–20	NA

[a]Date of infection was provided by the patient upon clinical examination. NA, non applicable.

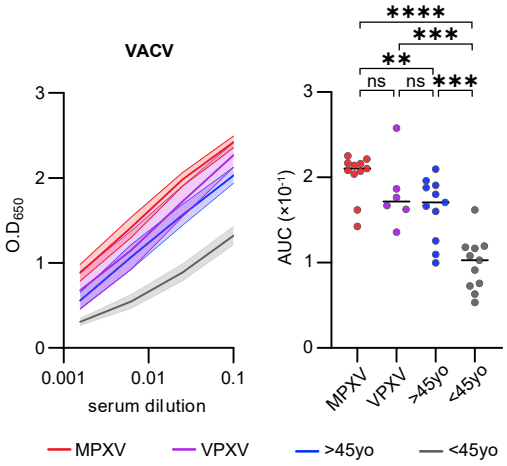
and fever (for 8 out of 11, Table 1). MPXV is highly similar to VACV, which is the prototype of this viral family.[13] Therefore, we first tested binding of heat-inactivated sera from MPXV recoverees to heat-inactivated VACV by enzyme-linked immunosorbent assay (ELISA) as previously described.[19] In parallel, we included sera samples of uninfected male donors, who were recently vaccinated against MPXV (Table S1) with live non-replicating Modified Vaccinia Ankara (JYNNEOS, n = 6, named "VPXV"). In addition, we included two volunteer groups; uninfected male donors below 45 years old (samples collected before the Monkeypox outbreak, n = 11, named "uninfected <45yo"), and uninfected male donors above 45 years old (samples collected before the Monkeypox outbreak, n = 11, named "uninfected >45yo"). The latter are expected to have been vaccinated in the past with a Vaccinia-based vaccine, as smallpox vaccination was standard protocol in Israel for individuals born until 1977. Heat-inactivated sera from all MPXV recoverees, and both recent and past vaccinated (i.e., VPXV and >45yo, respectively) reacted with VACV IHDJ strain (Figure 1A). The indistinguishable serological responses between recently vaccinated VPXV group and past vaccinated >45yo donors agrees with reports about the longevity of Vaccinia immunization.[20] Sera from uninfected <45yo donors had significantly lower binding to the inactivated VACV.

We next asked whether the donors exhibit neutralizing antibodies in their sera. Owing to the strict biosafety requirements for working with authentic MPXV, as a model we used Vaccinia Lister virus (VACV Lister) and determined neutralization of protein-A-purified IgG from MPXV recoverees in a plaque reduction neutralization test (PRNT). It has been already shown that VACV vaccination elicits serum neutralization against VACV Lister,[21,22] therefore, we focused on the MPXV recoverees' samples and tested whether they exhibit neutralization in this assay as well. Briefly, VACV Lister was pre-incubated with MPXV recoverees purified IgG and used to infect Vero cells. Purified IgG from uninfected donors <45yo were used as negative controls, whereas pooled purified IgG from multiple VACV vaccinated donors (Vaccinia immune globulin, 'VIG'[23]) was used as positive control. Surprisingly, only four MPXV recoverees exhibited any measurable neutralizing activity, with all of them having markedly less neutralization than VIG (Figures 1B and S1). It has been demonstrated that neutralization of both the MV and the EV forms of *Orthopoxviruses* requires the addition of complement.[24,25] Therefore, we repeated the assay while including complement serum. This time, additional four MPXV recoverees demonstrated measurable levels of neutralization (Figure 1B). For the four donors who exhibited neutralization without complement, neutralizing activity was improved by 10–44%, further emphasizing the importance of complement components in this assay. Nevertheless, even after complement serum was added, most of the donors demonstrated relatively weak neutralizing activity, with only one donor MPX03 exhibiting NT_{50}< 50 μg/mL. We conclude that although MPXV infection induces strong serological responses in ELISA, 33–62 days post infection, at this timepoint most recoverees do not exhibit strong neutralizing abilities against VACV Lister.

Sera and purified IgG from MPXV recoverees bind recombinantly expressed MPXV antigens

Next, we wished to map the targets for antibodies elicited following MPXV infection. We focused on three MPXV antigens that were previously described to be implicated in immune response against either MPXV

A



B

Patient ID	NT_{50} (μg/mL) - complement	NT_{50} (μg/mL) + complement
Mpx01	>300	>300
Mpx02	>300	82
Mpx03	77.5	43
Mpx04	177	119
Mpx05	>300	134
Mpx06	220	137
Mpx07	>300	>300
Mpx08	>300	219
Mpx09	84	75
Mpx10	>300	>300
Mpx11	>300	154
<45yo-1	NA	>300
<45yo-2	>300	>300
VIG	36	<15

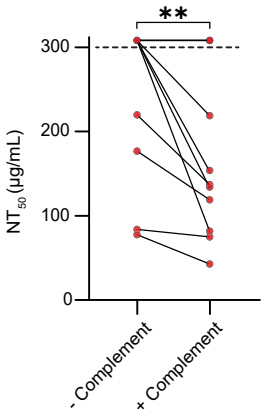


Figure 1. Sera from MPXV recoverees bind VACV and exhibit some VACV neutralization

(A) Left: binding curves as measured by ELISA at $O.D._{650nm}$ demonstrating serum response against VACV IHDJ strain. MPXV recoverees are in red (n = 11), VPXV are in purple (n = 6), uninfected >45yo are in blue (n = 11) and uninfected <45yo are in gray (n = 11). The mean serum response and standard error of the mean (SEM) are depicted in bold line and shadow, respectively, for each group. Four consecutive serum dilutions, starting from 1:10, for every group of donors were tested. Right panel: Area under the curve (AUC) values depicting each donor separately. Statistical analysis was performed using One-way ANOVA. **p< 0.01, ***p< 0.001, ****p< 0.0001.

(B) Left panel: NT_{50} values of purified IgG from 11 MPXV recoverees as measured in a PRNT using VACV Lister, with and without the addition of complement. Right panel: graphical representation of the NT_{50} values. Dashed line represents the PRNT's measurement cutoff values of 300 μg/mL. NA – non applicable. Statistical analysis was performed using Ratio paired t-test. **p< 0.01.

or VACV.[3,16–18,26,27] The transmembrane proteins were expressed in their soluble forms, i.e., without their corresponding transmembrane regions. Overall, we produced one antigen from the EV form, A35R (95.03% homologous to VACV antigen A33R) amino acids 58–181, and two antigens from the MV form: M1R (98.4% homologous to VACV antigen L1R) amino acids 2–185, and H3L (93.52% homologous to VACV antigen H3L) amino acids 2–277 (Figures 2A and 2B). The antigens were cloned into pcDNA3.1(–) expression vector, containing an 8xHistidine tag at their C-termini followed by Avi-tag, a 15-amino acid biotinylation sequence (Figure 2B). The antigens were expressed in mammalian Expi293F cells and purified on Nickel beads (Figure 2C).[28] *In silico* alignment of the AlphaFold-predicted structures of the recombinantly expressed MPXV antigen truncations to the atomic structures of their corresponding VACV homologs, predicted that MPXV and VACV antigens are likely to achieve similar conformations (Figure S2A).

The three purified antigens were used to coat ELISA plates and reacted with sera from MPXV recoverees, VPXV vaccinees, >45yo, and <45yo (Figure 2D). Sera from MPXV recoverees bound A35R in a dose dependent manner, and significantly stronger than all uninfected, including both vaccinated, groups. The second antigen, M1R, was recognized stronger by MPXV recoverees compared to uninfected <45yo, yet the response was similar to that of the two vaccinated groups (although this response was lower in the >45yo group). As to the third antigen, H3L, binding was not significantly different between MPXV and VPXV groups, however, the response was significantly higher in MPXV recoverees compared to both >45yo and <45yo groups (Figure 2D). The elite MPXV sera response to A35R and H3L was further demonstrated by the fact that the median reactivity to A35R and H3L was 2.5- and 2.1-fold higher, respectively, compared to M1R, which seems to be less immunodominant following infection (Figure S2B). This difference was not observed within the VPXV vaccinated sera, that bound similarly to all three antigens. Notably, A35R reactivity was significantly stronger within >45yo group and higher by 1.35-fold compared to M1R (Figure S2B). Similar results were obtained with protein-A-purified IgG (Figure 2E). Here too, the majority of MPXV recoverees bound A35R antigen stronger, even when compared to VIG positive control, with the median response of the MPXV group being higher than VIG throughout all IgG concentrations tested. We conclude that both A35R and H3L are targets for antibodies elicited following MPXV infection, with the response to the soluble 124-amino acid truncation of A35R being significantly higher in recently infected MPXV patients than in recently or past vaccinated individuals, who received a Vaccinia virus-based vaccine.

A

MPXV antigen	Full length a.a	VACV antigen	Full legth a.a	VACV PDB	%Aligment to VACV
A35R	181	A33R	185	3K7B	95.03
M1R	250	L1R	250	1YPY	98.4
H3L	324	H3L	324	5EJ0	93.52

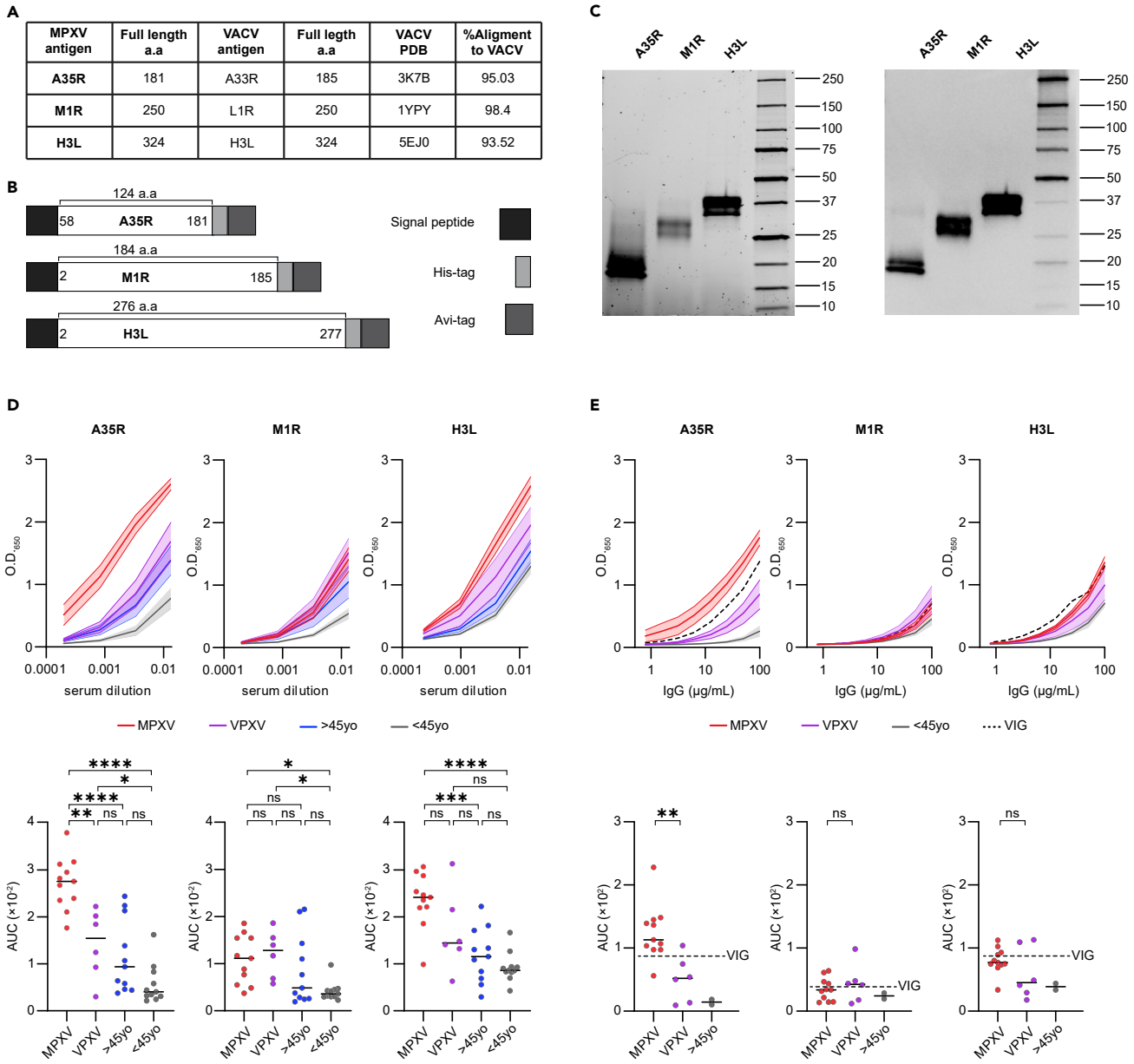


Figure 2. Sera and purified IgG from MPXV recoverees bind recombinantly expressed MPXV antigens

(A) Table listing the three MPXV antigens A35R, M1R and H3L produced in this study. Published PDB IDs of VACV homologs are given.[29–31] Amino-acid sequence alignment scores between MPXV and VACV antigens were calculated using the Clustal Omega web tool.[32]

(B) Construct design of the three recombinantly expressed antigens, A35R, M1R and H3L. Signal peptide, His-tag and Avi-tag are in black, light gray and dark gray, respectively. The amino acid section produced for each antigen is stated.

(C) SDS-PAGE (left) and western blot using mouse anti-Avi-tag antibody (right) of the recombinantly expressed MPXV antigens A35R, M1R and H3L after purification. The antigens are indicated on top, the protein marker is on the right of each gel/blot, and protein sizes are indicated on the right in kDa.

(D) Serum binding to MPXV antigens A35R, M1R and H3L as detected by ELISA. MPXV recoverees are in red (n = 11), VPXV are in purple (n = 6), uninfected >45yo are in blue (n = 11) and uninfected <45yo are in gray (n = 11). Upper panel: The bold lines and the shadowed areas on the graph represent the mean serum response and SEM, respectively, of 4 consecutive serum dilutions for every group of donors, starting from 1:75 dilution. Lower panel: AUC values for every individual donor. Statistical analysis was performed using One-way ANOVA. *p< 0.05, **p< 0.01, ***p< 0.001, ****p< 0.0001.

(E) Purified IgG binding to MPXV antigens A35R, M1R and H3L as detected by ELISA. MPXV recoverees are in red (n = 11), VPXV are in purple (n = 6), uninfected <45yo are in gray (n = 2), and VIG is represented by the dashed black line. Eight consecutive IgG dilutions starting from 100 µg/mL were tested (x axis). Upper panel: The bold lines and the shadowed areas on the graph represent the mean IgG response and SEM, respectively, for every group of donors. Lower panel: AUC values for every individual donor. Statistical analysis was performed between MPXV and VPXV groups using Welch's t test. **p< 0.01.

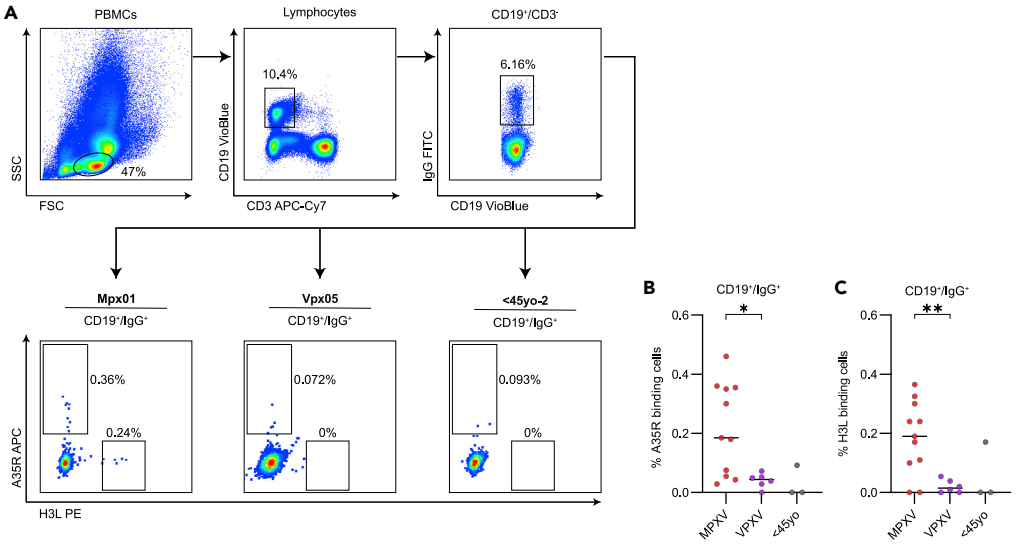


Figure 3. IgG$^+$ B cells from MPXV recoverees bind A35R and H3L antigens

(A) Flow cytometry gating strategy for staining of A35R- and H3L-specific IgG$^+$ B cells. Representative plots for MPXV recoveree (Mpx01), VPXV (Vpx05) and uninfected <45yo (<45yo-2) are shown.

(B) Frequency of A35R-specific IgG$^+$/CD19$^+$ cells of MPXV recoverees are in red (n = 11), VPXV are in purple (n = 6) and uninfected <45yo are in gray (n = 3). Statistical analysis was performed between MPXV and VPXV groups using Unpaired t test. *p< 0.05, **p< 0.01.

(C) The same as (B), but for H3L-specific IgG$^+$/CD19$^+$ cells.

IgG$^+$ B cells from MPXV recoverees bind A35R and H3L antigens

Antibodies are produced by B cells following antigen encounter and activation.[33] We therefore evaluated the ability of B cells collected from the 11 MPXV recoverees to specifically bind A35R and H3L antigens. For this purpose, 5–6 million PBMCs from whole blood were stained for CD3, CD19, IgG as described before,[28,34] as well as with biotinylated, streptavidin fluorophore conjugated A35R and H3L antigens (Figure 3A). We were able to record A35R-specific IgG$^+$/CD19$^+$ cells in all MPXV recoverees, frequencies ranging between 0.03% and 0.46% of the total IgG$^+$/CD19$^+$ cells (Figure 3B). Moreover, the levels of A35R-specific cells were significantly higher in MPXV recoverees compared to VPXV group. Similar levels of H3L-specific IgG$^+$/CD19$^+$ cells were detected. Although two donors did not exhibit any detectable H3L-specific IgG$^+$/CD19$^+$ cells, here too, the frequency of H3L-specific IgG$^+$/CD19$^+$ cells was significantly higher in MPXV recoverees compared to VPXV group (Figure 3C). MPXV recoverees and VPXV vaccinees exhibited similar frequencies of CD3$^+$ cells ranging from 42%-81%, CD19$^+$ cells ranging from 2%-12.7% and IgG$^+$ B cells (gated from CD19$^+$ population) ranging from 5%-30% (Figure S3). In this analysis we could not include samples from >45yo, as well as most <45yo, because for these samples PBMCs were not available.

DISCUSSION

In the present report we demonstrate that MPXV recoverees produce both antibodies and B cells against MPXV antigens A35R and H3L. The B cell response to both these antigens was higher compared to Vaccinia-based vaccinated donors. This might be because of slight differences between the two viruses, MPXV *versus* VACV. Another possibility is differences in the sequences of MPXV antigens and their VACV versions (A35R 95.03% homology and H3L 93.52% homology).

A35R, is one of the 6 proteins expressed on the EV form of the virus, which is believed to be responsible mostly for cell-to-cell viral spread.[35] Although the EV form of poxviruses is considered more protected and more difficult to neutralize by antibodies,[36] antibodies against EV proteins were found to mediate VACV neutralization after Vaccinia vaccination and MPXV infection[16,37] and removal of EV-directed antibodies abolished VIG neutralization.[38] Although most of the MPXV recoverees in our cohort did not exhibit high neutralizing activity against VACV in a plaque assay 1–2 months after infection, all of them bound a 124-amino acid truncation of A35R antigen, significantly stronger than did vaccinated or uninfected donors.

We found no correlation between A35R binding to MPXV sera and Vaccinia Lister neutralizing activity, which might be because of the slight differences between the two viruses, or because the plaque assay is mostly detecting MV-related viral inhibition, rather than EV-related viral inhibition. We found anti-A35R B cells in peripheral blood of all MPXV recoverees, at significantly higher levels than in vaccinated donors.

H3L antigen is expressed on the MV form, promoting binding to host cells and infectivity.[39] It was identified as a target for both T cells and B cells[17,27] in vaccinated mice and humans, and anti-H3L antibodies were able to elicit protection from a lethal challenge in mice.[40] In agreement with that, in our study, antibodies from MPXV recoverees and vaccinees (past and recent) bound H3L. MPXV recoverees also exhibited H3L-specific IgG^+ B cells, which were in higher frequency compared to recent vaccinees. Amongst the two MPXV recoverees who did not have any detectable H3L-specific IgG^+ B cells was donor Mpx06 who showed neutralizing activity against the VACV Lister in plaque assay, even without the addition of complement. This might suggest that other MPXV specific antibodies and T cells were elicited by infection and contributed to viral clearance.

Limitations of the study

Our study has several limitations; first, the MPXV samples were analyzed at a relatively early timepoint after Monkeypox infection, which could potentially result in low Vaccinia virus neutralization in PRNT. Furthermore, owing to the worldwide shortage of Monkeypox vaccines, only six samples from recently vaccinated donors were available. Moreover, these samples were collected after only one vaccine dose, and not the required two dose regimen (as well because of shortage in vaccine doses and Israeli Ministry of Health guidelines at the time the study was conducted). Another potential caveat of our work is the utility of the ELISA assay for the diagnosis of Monkeypox infections; although the response to the recombinantly expressed protein A35R was higher in all MPXV recoverees compared to both vaccinated groups, we could not rule out that this might be because of elevated antibody titers in the MPXV recoverees group, at the point of sample collection. More work is needed to determine the dynamics of the humoral response following Monkeypox infection.

STAR★METHODS

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

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.105957.

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AUTHOR CONTRIBUTIONS

R.Y. planned and performed the experiments, analyzed data, prepared the figures, and wrote the manuscript together with N.T.F. N.F. conducted the patient follow up and recruited the donors and collected the samples with the help of E.T. H.T. and T.I. carried out all the neutralization studies, analyzed viral inhibition data with the help of LCM, as well as critically reviewed the data and the manuscript. M.M. helped with protein production and cloning. K.P. performed the AlphaFold predictions. D.H. and E.S. wrote and submitted the ethics protocols, helped design the study and oversaw donor enrolment into the study. N.T.F. planned and supervised the experiments, wrote the ethical protocols, analyzed the data and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no conflict of interest.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-CD19 VioBlue	Miltenyi Biotec	Cat#130-113-172; RRID:AB_2725999
Anti-IgG FITC	Miltenyi Biotec	Cat#130-118-340; RRID:AB_2733672
Anti-CD3 APC-Cyanine7	Biolegend	Cat#317342; RRID:AB_2563410
Anti-IgG HRP	Jackson ImmunoResearch Labs	Cat#109-035-088; RRID:AB_2337584
Anti-Avi-tag	Avidity	Cat#AbC
Vaccinia immune globulin	Omrix	Cat#Omr-IgG-am ™ 5% IV
Bacterial and Virus Strains		
Vaccinia Lister	Israeli Ministry of Health	N/A
Vaccinia IHDJ	Prof. Ehud Katz	N/A
Biological Samples		
MPXV recoverees donors whole blood	This study	Helsinki approval number 0384-22-TLV
Recently vaccinated donors whole blood	This study	Helsinki approval number 0384-22-TLV
Uninfected healthy donors whole blood	Israeli Blood bank	protocol number 0004554–2
Guinea pig complement	Sigma-Aldrich	S-1639
Chemicals, Peptides, and Recombinant Proteins		
Streptavidin APC	Miltenyi Biotec	Cat#130-106-792; RRID:AB_2661578
Streptavidin PE	Miltenyi Biotec	Cat#130-106-789; RRID:AB_2661577
Ficoll-Paque PLUS	Cytiva	Cat#17-1440-03
Ni Sepharose beads	Cytiva	Cat#17-5318-01
Protein-A beads	Cytiva	Cat#17-5199-01
A35R	This study	N/A
M1R	This study	N/A
H3L	This study	N/A
Critical Commercial Assays		
MINI PLASMID PRESTO Kit	Geneaid	Cat#IMPDH300
NucleoSpin Plasmid Kit	MACHEREY-NAGEL	Cat#MAN-740588.250
Pierce™ BCA Protein Assay Kit	Thermo Scientific™	Cat#TS-23227
Experimantal Model: Cell Lines		
EXPI 293F	Thermo Scientific™	N/A
Vero cells	ATCC	ATCC-CCL-81
Recombinant DNA		
Truncated MPXV A35R sequence	This study/Genescript	N/A
Truncated MPXV M1R sequence	This study/Genescript	N/A
Truncated MPXV H3L sequence	This study/AZENTA	N/A
pcDNA3.1(–) plasmid	ThermoFisher Scientific	N/A
Software and Algorithms		
FlowJo v.10.8.1	FlowJo	https://www.flowjo.com/
GraphPad Prism v.9.4.1	GraphPad	https://www.graphpad.com/scientific-software/prism/
Biorender	Biorender.com	https://app.biorender.com/

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Adobe illustrator v.26.5	Adobe	https://www.adobe.com/il_en/products/illustrator.html
PyMOL v.2.5.2	PyMOL	https://pymol.org/2/
AlphaFold2	AlphaFold	https://alphafold.ebi.ac.uk/
SnapGene v.6.0.2	SnapGene	https://www.snapgene.com/
Other		
BirA 500 Kit	Avidity	Cat#BirA500; Lot: B12920
Monkeypox reference genome 2018	NCBI GenBank	Accession no. MN648051
ExpiFectamine™ 293 Transfection Kit	Gibco™	Cat#A14524

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Natalia T Freund (nfreund@tauex.tau.ac.il).

Materials availability

Monkeypox virus antigens generated in this study will be available upon request from the lead contact with a completed Materials and Transfer Agreement.

Data and code availability

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

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Ethics statement

All donors provided a written informed consent prior to participating in this study. All participants enrolled in this study were males. MPXV recoverees between the ages of 23 and 39 tested positive in a PCR assay for MPXV infection collected from saliva, blood, anus, or blisters. All 11 MPXV recoverees have not received a Vaccinia virus (VACV) based vaccine. A single sample of 170 mL of whole blood was collected 33–62 days post infection, after the donors were considered recovered. Recently vaccinated donors (VPXV) were recruited 26–39 days after receiving a single dose of the JYNNEOS vaccine. A single sample of 30 mL of whole blood was collected. Tel Aviv University Institutional Review Board (IRB) approved all studies involving patient enrollment, sample collection, and clinical follow-up (protocol number 0005243–1). Donors were followed by the Dermatology Division of Ichilov Tel Aviv Sourasky Medical Center (Helsinki approval number 0384-22-TLV). The uninfected >45yo and uninfected <45yo samples were collected from the Israeli Blood bank (protocol number 0004554–2). Both <45yo and >45yo samples were obtained between 2020-2021, before the current Monkeypox outbreak. <45yo donors are not expected to have been previously vaccinated with a VACV based vaccine.

Viruses

VACV-Lister (Elstree; provided by the Israeli Ministry of Health) was propagated on the chorioallantois membranes of embryonated eggs and titrated on Vero cells (ATCC-CCL-81). Vaccinia IHDJ strain was kindly provided by Prof. Ehud Katz.

METHOD DETAILS

Sample collection and processing

Blood samples of 170 mL were collected at least two weeks after MPXV recovery, and 30 mL at least 26 days post vaccination. One VPXV donor (VPXV04) received a VACV based vaccine prior to the current MPXV

outbreak. All samples were processed for PBMCs isolation. Briefly, each whole blood sample was diluted 1:3 in RPMI 1640 medium and separated using Ficoll gradient according to the manufacturer's protocol. Buffy-coat layer cells were washed 3 times with RPMI, resuspended in FBS containing 10% DMSO and frozen in liquid nitrogen until later use.

Expression of MPXV antigens

Viral isolate sequenced in Israel from the 2018 MPXV outbreak, NCBI GenBank (accession no. "GeneBank: MN648051"),[2] was used as a template for MPXV antigen production. The DNA sequences of the soluble domains of antigens A35R, M1R and H3L were optimized for expression in mammalian cells and added to an 8×Histidine sequence (His-tag) and biotinylation encoding sequence (Avi-tag), before being sent to an outsider vendor for production. Generated DNA sequences were cloned into the pcDNA3.1(−) vector and transfected into EXPI293F cells. Expressed antigens were purified from the cells medium using Nickel beads.[28,41] Antigens concentration for subsequent tests was measured using NanoDrop Spectrometer at $O.D._{280nm}$ and BCA protein assay.

SDS-PAGE and western blot

To validate proper expression of the MPXV antigens, SDS-PAGE was performed using 1 μg of each antigen containing 5% β-mercaptoethanol. Western Blotting was performed by transferring the proteins to a Nitrocellulose membrane followed by blocking the membrane for 2 hours at room temperature (RT) in blocking buffer containing PBS×1, 3% BSA, 0.05% Tween20 and 20mM EDTA. The membrane was incubated overnight at 4° with a mouse anti-Avi-tag antibody diluted 1:5000 in blocking buffer. After 5 washes with washing buffer containing PBS×1 and 0.05% Tween20, the membrane was incubated with a secondary HRP-conjugated antibody diluted 1:5000 in blocking buffer for 45 minutes at RT. Following 5 additional washes, ECL was added, and Western blot images were acquired.

Plaque reduction neutralization test

MPXV recoverees neutralization activity was assessed in a PRNT. IgG from all MPXV recoverees and from 2 uninfected <45yo donors was purified using protein-A agarose beads. For PRNT without complement, 50 plaque forming units (PFU) per well of VACV Lister were pre-incubated for 1 hour with MPXV or <45yo purified IgG at a starting concentration of 200 μg/mL followed by 6 consecutive 2-fold dilutions. Vaccinia immune globulin, 'VIG', was also used as positive control at a starting concentration of 500 μg/mL followed by 6 consecutive 2-fold dilutions. Pre-incubated Vaccinia and purified IgG were used to infect 5×10^5 Vero cells. The experiment was carried out in duplicates, and 72 hours post infection plaques were counted and NT_{50} was determined. For PRNT with complement, Guinea pig complement (S-1639) was added to the Vaccinia Lister, reaching a final concentration of 1% after pre-incubation with purified IgG. Subsequent steps of the PRNT with complement are similar.

Enzyme-linked immunosorbent assay

VACV ELISA: VACV IHDJ strain was diluted to a concentration of 10^7 PFU per mL in a carbonate bicarbonate solution and used to coat high binding ELISA plates overnight at 4°. ELISA plates were then blocked for 2 hours at RT in a TSTA solution containing 2% BSA, 50mM Tris, 142mM NaCl, 0.05% Azid and 0.05% Tween20 followed by washing the plates once in washing buffer containing PBS×1 and 0.05% Tween20. Heat inactivated sera samples were diluted 4-fold in TSTA, starting from 1:10 and added to the ELISA plates for 1 hour at RT. ELISA plates were washed 3 times with washing buffer followed by adding anti-IgG HRP-conjugated antibody diluted 1:5000 in TSTA for 45 minutes at RT. Following 5 additional washes, TMB/E was added, and the optical density (OD) was measured after 10 minutes at 650 nm.

MPXV antigen ELISA: 5 μg/mL of each of the purified antigens, A35R, M1R and H3L, were used to coat high binding ELISA plates overnight at 4°. ELISA plates were then blocked for 2 hours at RT in blocking buffer containing PBS×1, 3% BSA, 0.05% Tween20 and 20mM EDTA followed by washing the plates once in washing buffer containing PBS×1 and 0.05% Tween20. Heat inactivated sera samples or purified IgG were diluted 4- and 2-fold in blocking buffer starting from 1:75 and 100 μg/mL, respectively, and added to the ELISA plates for 1 hour at RT. ELISA plates were washed 3 times with washing buffer followed by adding anti-IgG HRP-conjugated antibody diluted 1:5000 in blocking buffer for 45 minutes at RT. Following 5 additional washes, TMB/E was added, and the OD was measured after 10 minutes at 650 nm.

Flow cytometry

5–6 million PBMC were thawed at 37°, washed in RPMI and resuspended in FACS buffer containing PBS×1, 1% FBS and 2mM EDTA. For antigen-specific B cell staining, MPXV antigens A35R and H3L were biotinylated on their Avi-tag sequence using the BIR-A biotinylation reaction, followed by their conjugation to streptavidin coupled fluorophores. The PBMCs were stained for CD3, CD19, IgG as well as for with biotinylated, streptavidin fluorophore conjugated A35R and H3L and analyzed in a Flow Cytometer.

Statistical and Flow Cytometry analysis

Statistical analysis was carried out using the GraphPad Prism software version 9.4.1. Analysis of Flow Cytometry data was carried out using FlowJo software version 10.8.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis was carried out using the GraphPad Prism software version 9.4.1. Analysis of Flow Cytometry data was carried out using FlowJo software version 10.8. Statistical details of each experiment can be found in the figure legend associated with each figure.