The impact of orthopoxvirus vaccination and Mpox infection on cross-protective immunity: a multicohort observational study

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Summary

Background Cross-reactive immune memory responses to orthopoxviruses in humans remain poorly characterised despite their relevance for vaccine design and outbreak control. We aimed to assess the magnitude, specificity, and durability of cross-reactive immune responses elicited by smallpox vaccines and mpox virus infection.

Methods We did a multicohort observational study involving participants from the USA, Brazil, and Portugal across four groups: Dryvax (first-generation smallpox vaccine) recipients vaccinated 40–80 years ago, JYNNEOS (third-generation smallpox vaccine) recipients vaccinated within the past year, a cohort receiving both vaccines, and patients infected with clade IIb mpox. Samples were analysed for systemic and mucosal humoral responses, neutralising antibody titres, viral antigen structural analysis, and T-cell cross-reactivity to vaccina virus, cowpox virus, and mpox virus. Statistical analyses included correlation assessments and comparisons across cohorts to determine the magnitude, longevity, and breadth of immune responses.

Findings Between July 7, 2022, and Aug 3, 2023, 262 participants were recruited, resulting in analysis of 378 samples. Both first-generation and third-generation smallpox vaccines elicited vaccinia virus-reactive and mpox virus-reactive antibodies, with the strongest responses targeting the less conserved extracellular virion antigens B5 and A33. Despite high concentrations of anti-mpox virus antibodies in the plasma, cross-neutralisation activity correlated with viral antigenic distance. Higher neutralisation was observed for cowpox virus than for mpox virus, which has lower antigenic conservation with vaccina virus. Complement-mediated neutralisation enhanced mpox virus neutralisation, overcoming the limitations of antigenic distance. Dryvax recipients sustained vaccina virus neutralisation titres for over 80 years, whereas cross-reactive responses did not show this durability. JYNNEOS-induced responses waned within a year. T-cell cross-reactivity was long-lasting, detected up to 70 years after vaccination. Booster vaccinations augmented the magnitude, breadth, and longevity of cross-neutralising responses.

Interpretation Our findings highlight the potential combined role of antibody effector functions and T-cell memory in cross-protection against orthopoxviruses. Complement-mediated neutralisation enhances cross-protection, overcoming antigenic distance. These Fc-mediated functions, along with T-cell responses, contribute to effective and long-lasting immunity conferred by smallpox vaccines against other orthopoxviruses.

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Introduction

The smallpox vaccine is the only vaccine that has led to the eradication of a human disease.[1,2] Despite this success, multiple questions regarding protective mechanisms, cross-protection against other orthopoxviruses, and the durability of immune memory responses remain unanswered. Specifically, the mobilisation of cross-reactive memory cells into a primary immune response, and the induction and maintenance of this response over time in comparison to an immunodominant primary response, are not fully understood.

Individuals born before 1980 received smallpox vaccination under the smallpox eradication campaign (1966–80).[3] This campaign used a variety of live-attenuated strains of vaccinia virus (VACV), leading to successful eradication of smallpox and potentially generating broad cross-protective immunity against additional orthopoxviruses, including mpox virus.[3] Mpox, previously classified primarily as a zoonotic disease caused by mpox virus, has emerged as the most common orthopoxvirus that infects humans since smallpox eradication. Mpox virus has shown increasing human-to-human transmission and varying levels of virulence and lethality across different viral clades, making it a substantial and evolving public health threat. Although initially endemic to central and western Africa, recent

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Research in context

Evidence before this study

We reviewed existing literature on immunogenicity and cross-protection mechanisms of smallpox vaccines and mpox virus infection before this study. We searched PubMed and Scopus with the terms "smallpox vaccine", "orthopoxvirus immunity", "mpox virus infection", "orthopoxvirus cross-reactive immune responses", and "vaccine-induced T cell responses", from database inception to July 13, 2024. Previous research has examined the immunogenicity of first-generation (Dryvax) and third-generation (JYNNEOS) smallpox vaccines and their cross-reactivity to orthopoxviruses, including mpox virus. Although long-term humoral responses to Dryvax have been shown to persist for decades, evidence on the durability and breadth of cross-reactive immune responses, particularly against antigenically distant viruses, such as mpox virus, remains scarce. Additionally, there is little understanding of the roles of T-cell-mediated immunity and antibody effector functions, such as complement-mediated neutralisation, in orthopoxvirus cross-protection.

Added value of this study

Our study expands on previous findings by providing a detailed analysis of immune responses across multiple cohorts, encompassing diverse smallpox vaccination regimens (first-generation, third-generation, and combination boosters) and mpox virus infection. By including participants ranging from 7 days to over 80 years since vaccination, we capture both short-term and long-term immunity. Our analyses span systemic and mucosal responses, evaluating both humoral and T-cell-mediated immune responses. We show that first-generation and third-generation smallpox vaccines elicit robust, long-lasting T-cell responses but restricted and transient cross-neutralising antibody responses against mpox virus. This study correlates viral antigenic distance with diminished neutralisation capacity, while highlighting the compensatory role of Fc-mediated antibody effector functions, such as complement recruitment, in overcoming this limitation. Furthermore, our structural analyses of viral antigens reveal specific mutations that might hinder antibody binding, providing insights into mechanisms underlying cross-protective immunity. These findings address key knowledge gaps regarding the durability and breadth of vaccine-induced immunity and the mechanisms contributing to orthopoxvirus cross-protection.

Implications of all the available evidence

Our findings show the need to improve vaccine designs to target more conserved antigens for broader and longer-lasting protection against orthopoxviruses. Our results suggest that additional boosters can further enhance the magnitude and breadth of the immune response. Incorporating strategies to boost antibody effector functions and durable T-cell immunity could mitigate the limitations imposed by antigenic distance. These insights are crucial for informing vaccine development, particularly in light of the ongoing threat posed by emerging mpox virus strains. Future research should focus on refining vaccine strategies, exploring novel platforms, such as mRNA vaccines, and assessing vaccine ability to elicit robust, long-lasting, and broad-spectrum immunity. Additionally, understanding the dynamics of cross-reactive immune memory and implications for vaccine-boosting strategies will be crucial for effectively addressing future orthopoxvirus outbreaks.

outbreaks have shown changes in both transmission patterns and clinical presentations, raising concerns globally.[4,5] Specifically, in summer 2022, there was a multicountry outbreak of clade IIb mpox virus, which primarily affected gay, bisexual, or other men who have sex with men. This outbreak was characterised by transmission occurring primarily in sexual settings, resulting in genital and rectal lesions at the site of exposure.[6,7] By contrast, clade I mpox virus, traditionally reported in children in west and central Africa, with infections often linked to animal contact or bushmeat consumption and leading to sporadic household transmission,[8] has shown a rapid and concerning spread as of summer 2024. The Democratic Republic of the Congo and other African countries have reported a surge in mpox cases and, given its high fatality rate of 4–11%, increased transmissibility, and rising case numbers, WHO declared the ongoing mpox virus clade Ib outbreak a public health emergency of international concern in August, 2024.

Smallpox vaccines provide a valuable model to study immune response longevity, as typically individuals are not repeatedly exposed. However, the mechanisms underlying memory maintenance, particularly the persistence of cross-reactive memory B cells during an immunodominant primary response, remain unclear. Given the high genetic similarity among poxviruses, it has long been assumed that smallpox vaccines can confer broad cross-protection. However, the protective limits conferred by smallpox vaccines and the mechanisms underlying cross-protective immunity remain poorly understood. The first-generation, live-attenuated vaccinia virus vaccine for smallpox, Dryvax (Wyeth Laboratories; Collegeville, PA, USA), was formulated using the vaccinia virus-New York City Board of Health (NYCBH) strain in the USA but was discontinued due to safety concerns in 2001.[9] Dryvax was replaced by ACAM2000 (Emergent Biosolutions; Gaithersburg, MD, USA), a second-generation vaccine derived from vaccinia virus-NYCBH and produced in cell line culture. However, ACAM2000 is also associated with serious side-effects, such as encephalitis and myocarditis, and is not recommended for use in immunosuppressed individuals and those with atopic skin disease. The third-generation vaccine, JYNNEOS (Bavarian Nordic; Hellerup, Denmark), uses a safer modified vaccinia Ankara virus. This strain, attenuated in avian cells, shows reduced virulence and replication capabilities in mammals, making JYNNEOS use safe for

immunocompromised patients.[10] Although JYNNEOS was reported to have an 86% effectiveness (95% CI 59–95) for preventing mpox disease shortly after vaccination and similar estimates after the second dose, detailed insights into the immunogenicity and longevity of its protective responses in humans remain scarce.[11,12] Although JYNNEOS has been used in the USA and Europe, its laborious production and high cost limit widespread distribution, particularly in Africa, where mpox virus is spreading rapidly. Similarly, new mpox virus-specific mRNA vaccines have shown promise due to high immunogenicity and protection in macaques; however, these vaccines still need to be evaluated in humans, and their costly production will likely hinder global distribution. In this study, we aimed to investigate the magnitude and breadth of cross-protective immunity conferred by accessible smallpox vaccines against new emerging orthopoxvirus threats. Decoding these dynamics could inform vaccine design, leading to the development of more potent vaccines, targeting a broader range of pathogens.

Methods

Study design and participants

We did a multicohort observational study to investigate the magnitude, duration, and cross-reactivity of orthopoxvirus-induced immune responses. Participants were recruited from the USA, Brazil, and Portugal and categorised into four cohorts based on their vaccination or infection status (figure 1A). 271 participants provided 378 samples, including plasma, peripheral blood mononuclear cells (PBMCs), saliva, and rectal swabs. Participants were recruited based on their vaccination status (ie, previously vaccinated with Dryvax or intending to receive JYNNEOS vaccination). Individuals who reported previous infection with vaccinia virus or mpox virus or had close contact with infected people were excluded. Demographic data were gathered via a screening questionnaire at blood collection and electronic health records. Sex data were self-reported, with participants selecting between male, female, or prefer not to say.

In cohort 1 (Dryvax), 87 individuals aged 40–90 years who received a single dose of the first-generation smallpox vaccine, Dryvax, 40–80 years ago were included. The median age was 61 years (IQR 52–70), and 59 (68%) of 87 participants were female. Participants reported no previous infection or direct contact with infected patients. Participants with more than one vaccination dose were excluded. Plasma and PBMCs were collected at a single timepoint.

In cohort 2 (JYNNEOS), 62 individuals older than 18 years who had been vaccinated within the past year with the third-generation smallpox vaccine, JYNNEOS, in a two-dose regimen were included. The median age was 29 years (IQR 26–34) and 24 (39%) of 62 participants were female. Samples were collected at baseline (before vaccination), 7 days and 30–60 days after the first dose, 7 days and 30–60 days after the second dose, and 210–335 days after the first dose.

In cohort 3 (combination cohort), 29 individuals older than 40 years who received both Dryvax (40–80 years ago) and JYNNEOS (between July 7, 2022, and Aug 3, 2023) were included, with sampling mirroring cohort 2. The median age was 56 years (IQR 52–60) and 19 (66%) of 29 participants were female.

In cohort 4 (mpox clade IIb), 68 patients who were convalescent with mpox were included, with samples collected 2–80 days after symptom onset. The median age was 33 years (IQR 29–41) and two (3%) of 68 participants were female. Most individuals in this cohort were immunosuppressed (56 [82%] of 68 were HIV-positive, reflecting the demographics most affected by the 2022 clade IIb mpox outbreak). Smallpox vaccination status for many participants in this cohort was unknown, and individuals were classified as either vaccinated versus unvaccinated or unsure. Some analyses were done only with individuals confirmed as unvaccinated.

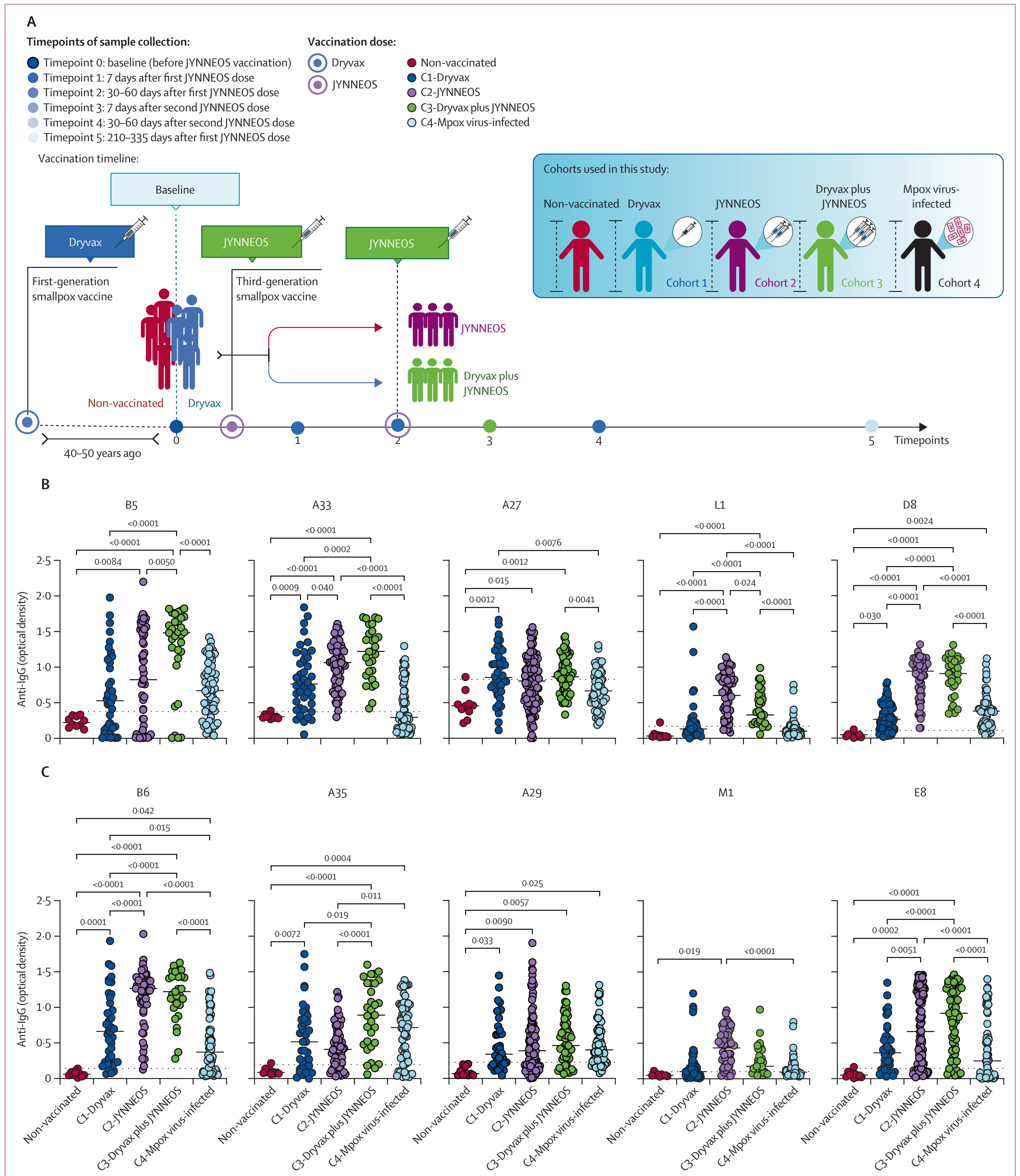
The study groups were stratified based on the vaccination regimen, infection status, sex, and age. Non-infected, non-vaccinated individuals were used as controls. Smallpox vaccinations were discontinued in the 1980s; therefore, participants in cohorts 1 and 3 are aged 40 years and older. By contrast, cohort 2 primarily consisted of young men, aligning with the target demographic for JYNNEOS vaccination. To reduce the potential confounding effects of age in our initial analysis, we focused on a subset of younger adults (aged 40–59 years) within cohorts 1 and 3. The full cohort 1 was exclusively analysed in the long-term immunological memory analysis, which involved only participants from cohort 1 who received Dryvax. Demographic and clinical data were obtained via questionnaires, electronic health records (EPIC EHR), and REDCap 9.3.6 (appendix p 5). This study was approved by the Yale Human Research Protection Program institutional review board (protocol ID 2000033415), the Research Ethics Committee of the Hospital Universitário Clementino Fraga Filho Universidade Federal do Rio de Janeiro (protocol number CAAE 62281722.5.0000.5257), and the Portuguese General Directorate of Health, through the technical orientation number 004/2022 on May 31, 2022. Written informed consent was obtained from all enrolled vaccinated or infected individuals.

See Online for appendix

Procedures

Whole blood was collected in heparinised CPT vacutainers (BD Biosciences; Franklin Lakes, NJ, USA) and processed on the same day. Plasma was isolated and stored at –80°C. PBMCs were extracted, treated with ammonium–chloride–potassium lysis buffer, and cryopreserved for further analysis.

Plasma orthopoxvirus-specific and mucosal IgA antibodies were quantified using ELISA. MaxiSorp plates (Thermo Fisher Scientific; Waltham, MA, USA) were coated with orthopoxvirus antigens or total IgA capture antibodies. Plasma, saliva, and rectal swab samples were



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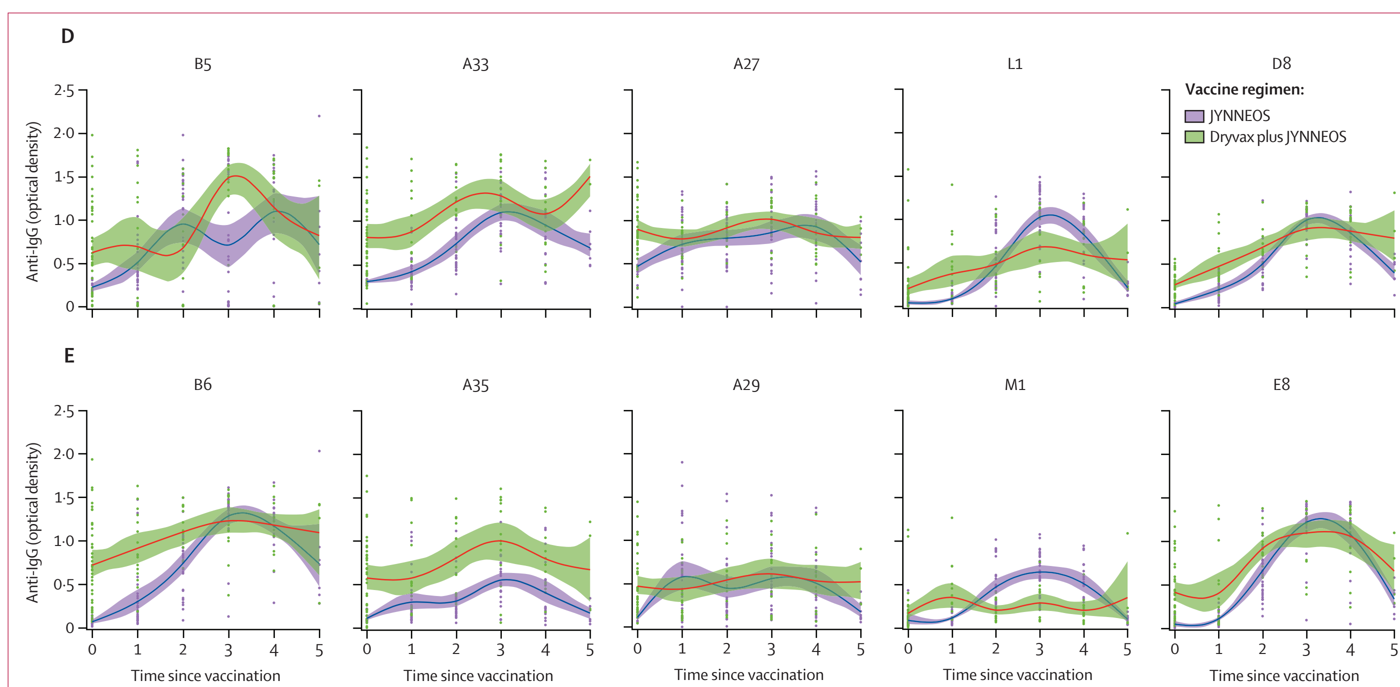


Figure 1: **Characterisation of orthopoxvirus-reactive antibody responses after first-generation or third-generation (or both) poxvirus vaccines**
(A) Cohort timeline overview showing time since vaccination and vaccine regimen received. The study included individuals vaccinated with Dryvax, JYNNEOS, or both Dryvax and JYNNEOS vaccines, and patients infected with clade II mpox virus. Plasma samples were collected at the indicated timepoints, and participants were stratified into four cohorts, versus a non-vaccinated control group: non-vaccinated (n=25); Dryvax (cohort 1; n=87); JYNNEOS (cohort 2; n=62); Dryvax plus JYNNEOS (cohort 3; n=29); mpox virus-infected patients (cohort 4; n=68). Dryvax was administered in a single-dose regimen at least 40 years before the study, whereas the JYNNEOS regimen comprised two vaccination doses and was recently administered to these participants. Figure created with BioRender.com. Plasma reactivity IgG to viral antigens was measured across all fully vaccinated individuals, patients infected with mpox virus, and non-vaccinated controls. Individual points represent individual participants. (B) Plasma reactivity to vaccinia virus proteins B5, A33, A27, L1, and D8. (C) Plasma reactivity to clade Ia mpox virus proteins B6, A35, A29, M1 and E8. In parts B and C, median IgG titre is represented within each group by horizontal bars; cutoffs were defined by mean plus 2 SDs from non-vaccinated controls and are represented by dashed lines. (D, E) Locally weighted scatterplot smoothing regression analysis of virus-reactive IgG concentrations over time after JYNNEOS vaccination. Individual points represent individual participants. (D) Plasma reactivity to vaccinia virus proteins. (E) Plasma reactivity to mpox virus proteins. Shading represents 95% CIs. For cohort 2, baseline controls comprised non-vaccinated individuals. Baseline controls for cohort 3 were individuals who had previously been vaccinated with Dryvax. For the x-axis labels in parts D and E, 0 indicates baseline, 1 indicates 7 days after first vaccine dose, 2 indicates 30–60 days after first vaccine dose, 3 indicates 7 days after second vaccine dose, 4 indicates 30–60 days after second vaccine dose, and 5 indicates 210–335 days after first vaccine dose.

diluted serially, incubated, and developed with 3,3′,5,5′-tetramethylbenzidine substrate. Optical density was measured at 450 nm.

PBMCs were stimulated in vitro with previously described orthopoxvirus-specific or mpox-specific peptide megapools[13] and cultured for 9 days with cytokine supplementation. Flow cytometric analysis of virus-specific T cells was done using an Attune NXT cytometer (Thermo Fisher Scientific; Waltham, MA, USA) and analysed with FlowJo version 10.6.

Vaccinia virus strains (VV.NP-S-EGFP and Lederle-Chorioallantoic), mpox virus (hMPXV/USA/MA001/2022), and cowpox virus (Brighton Red) were obtained from BEI Resources (NR-727, NR-56, NR-53, NR-624, NR-58622, and NR-88) and expanded in HeLa or BS-C-1 cells. The re-sequenced genomes were submitted to the National Center for Biotechnology Information (GenBank accession numbers U94848, ON563414, and NC_003663). Virus titres were determined by plaque assay.

Neutralisation capacity was assessed by plaque reduction neutralisation tests with or without complement. Plasma was serially diluted and incubated with orthopoxviruses before infecting susceptible cells. Both direct and complement-mediated neutralisation assays were always done with the respective viral controls, using established viral concentrations to generate 60–120 plaques per well (appendix p 12).

Poxvirus antigen sequences were aligned using Clustal Omega. Antigen–antibody interactions were visualised with PyMol version 2.5 and SWISS homology models based on vaccinia antigen protein data bank templates.

Statistical analysis

Data were analysed using GraphPad Prism version 10, JMP version 15, and R version 4.3.1. Group comparisons were assessed using ANOVA with Tukey's or Dunnett's corrections for multiple comparisons. Non-parametric tests (Mann-Whitney) were used for single-group comparisons. Locally weighted scatterplot smoothing regression was used for antibody kinetic analysis. $p<0·05$ was considered to indicate a statistically significant difference.

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

	Samples (n=378)	Mean (SD)	Participants (n=271)	p value
Dryvax cohort				
Age, years	··	51·75 (6·27)	··	··
40–49	13/40 (33%)	45·30 (2·90)	13/40 (33%)	<0·0001
50–59	25/40 (63%)	54·72 (3·26)	25/40 (63%)	··
60–69	2/40 (5%)	61·50 (0·71)	2/40 (5%)	··
Sex				
Male	10/40 (25%)	··	10/40 (25%)	··
Female	30/40 (75%)	··	30/40 (75%)	··
Dryvax cohort—extended full cohort				
Age, years	··	61·09 (12·15)	··	··
40–49	17/88 (19%)	44·29 (3·63)	17/87 (20%)	<0·0001
50–59	25/88 (28%)	54·72 (3·26)	24/87 (28%)	··
60–69	22/88 (25%)	64·68 (2·47)	22/87 (25%)	··
70–79	14/88 (16%)	72·64 (2·64)	14/87 (16%)	··
≥80	10/88 (11%)	81·50 (1·51)	10/87 (11%)	··
Sex				
Male	25/88 (28%)	··	25/87 (29%)	··
Female	59/88 (67%)	··	58/87 (67%)	··
No data	4/88 (5%)	··	4/87 (5%)	··
Mucosal samples				
Saliva	0	··	0	··
Rectal swab	4/88 (5%)	··	4/87 (5%)	··
JYNNEOS cohort				
Age, years	··	30·40 (6·78)	··	··
20–29	75/136 (55%)	25·33 (2·20)	32/62 (52%)	<0·0001
30–39	47/136 (35%)	33·91 (3·22)	26/62 (42%)	··
40–49	14/136 (10%)	43·36 (2·02)	8/62 (13%)	··
Sex				
Male	72/136 (53%)	··	34/62 (55%)	··
Female	59/136 (43%)	··	24/62 (39%)	··
No data	5/136 (4%)	··	4/62 (6%)	··
Time post-vaccination				
Baseline	12/136 (9%)	··	··	··
Dose 1 7–10 days	31/136 (23%)	··	··	··
Dose 1 30–60 days	31/136 (23%)	··	··	··
Dose 2 7–10 days	30/136 (22%)	··	··	··
Dose 2 30–60 days	22/136 (16%)	··	··	··
Dose 2 ≥180 days	10/136 (7%)	··	··	··
Vaccination interval, days	··	43·14 (8·79)	··	··
Regular (<40)	21/136 (15%)	28·60 (0·97)	27/62 (44%)	··
Extended (≥41)	45/136 (33%)	46·23 (6·17)	35/62 (56%)	··
Mucosal samples				
Saliva	13/62 (21%)	··	13/62 (21%)	··
Rectal swab	14/62 (23%)	··	14/62 (23%)	··
JYNNEOS and Dryvax cohort				
Age, years	··	54·76 (5·72)	··	··
40–49	11/61 (18%)	47·09 (0·3)	6/29 (21%)	<0·0001
50–59	32/61 (52%)	55·84 (2·82)	14/29 (48%)	··
60–69	16/61 (26%)	62·94 (2·62)	7/29 (24%)	··
70–79	1/61 (2%)	70·00 (0)	1/29 (3%)	··
Sex				
Male	23/61 (38%)	··	10/29 (34%)	··
Female	38/61 (62%)	··	19/29 (66%)	··
Time post-vaccination				
Baseline	88/88 (100%)*	··	··	··
Dose 1 7–10 days	16/61 (26%)	··	··	··

(Table continues on next page)

Results

Between July 7, 2022, and Aug 3, 2023, 262 participants were recruited from the USA (Yale New Haven Hospital, New Haven, CT, USA), Brazil (Federal University of Rio de Janeiro, Rio de Janeiro, Brazil), and Portugal (Hospital Curry Cabral, Lisbon, Portugal) to explore the magnitude, duration, and cross-reactivity of orthopoxvirus-induced immune responses, resulting in analysis of 378 samples. Participants were categorised into four cohorts. The first two cohorts comprised individuals vaccinated with either first-generation (Dryvax) or third-generation (JYNNEOS) generation smallpox vaccines, and the third cohort had received a combination of both vaccines. The fourth cohort comprised patients infected with clade IIb mpox. Non-vaccinated, non-infected individuals were used as controls. Detailed demographics are provided in the table.

Plasma antibody reactivity against vaccinia virus and clade Ia mpox virus antigens was measured in fully vaccinated individuals and patients infected with mpox virus. Orthopoxviruses have two distinct infectious forms: the mature virion and the extracellular virion. Antibody levels were assessed against key VACV recombinant proteins, B5, A33, A27, L1 and D8, present on both viral forms and previously identified as main targets of neutralising antibodies.[14–16] Detectable anti-vaccinia virus IgG levels were observed in 34 (85%) of 40 vaccinated participants in cohort 1, 61 (98%) of 62 vaccinated participants in cohort 2, and 29 (100%) of 29 vaccinated participants in cohort 3. The highest antibody titres targeted extracellular virion antigens B5 and A33, with peak levels observed in participants vaccinated with Dryvax and boosted with JYNNEOS (cohort 3; figure 1B). No differences were observed in antibody levels between vaccinated participants of different sexes (appendix p 6). IgG levels to mpox virus orthologous antigens B6, A35, A29, E8, and M1 were detected across all vaccinated cohorts, indicating humoral cross-reactivity between vaccinia virus and mpox virus antigens (figure 1B, C). The most pronounced increase in anti-mpox virus IgG antibodies was observed in cohort 3 (figure 1C). Patients infected with clade IIb mpox virus (cohort 4) showed detectable antibody levels reactive to both mpox virus and vaccinia virus proteins (figure 1B, C). Vaccination status for cohort 4 was unknown, and participants might have received smallpox vaccination before mpox virus infection. Moreover, although vaccine cohorts reflect peak antibody levels, mpox virus-infected samples were collected over a broad time range, possibly affecting antibody analysis. Longitudinal analysis of cohorts 2 and 3 revealed a peak IgG level between 7 days and 30 days after the second JYNNEOS dose (figure 1D, E). As expected, virus-reactive IgG concentrations were higher against vaccinia virus antigens compared with the cross-reactive mpox virus orthologues (appendix p 6). Antibody levels were dose-dependent, with higher levels observed after the second JYNNEOS dose (appendix p 7). Notably, antibody responses in Dryvax and JYNNEOS-boosted individuals declined over time for all antigens except L1, returning to baseline levels within 6–11 months after JYNNEOS booster (figure 1D, E;

appendix p 7). Overall, these observations indicate that both first-generation and third-generation vaccines effectively induce antibody responses against VACV and monkeypox virus antigens. However, JYNNEOS-induced antibodies wane significantly, returning to baseline approximately one-year post-vaccination.

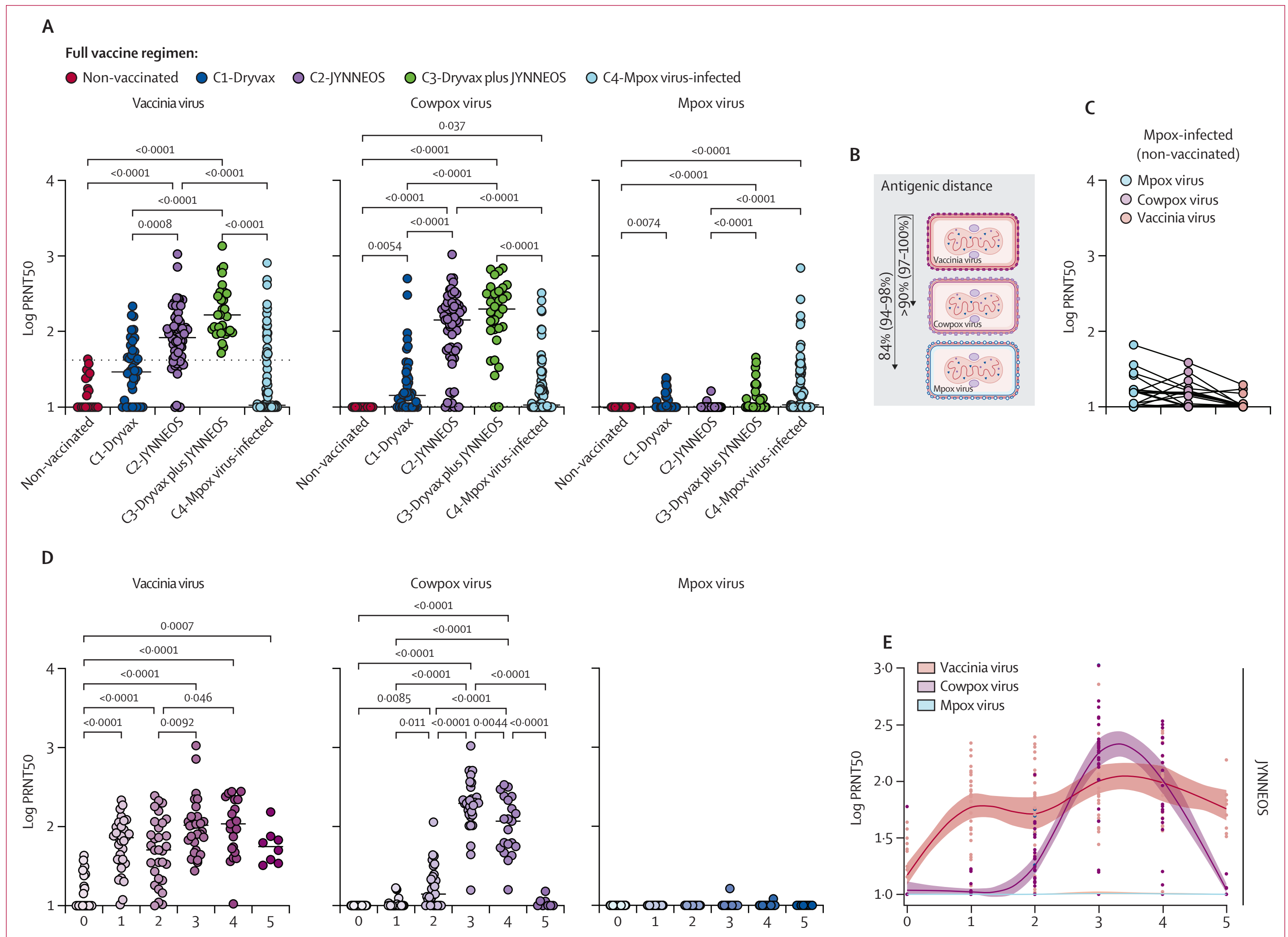
Orthopoxviruses are primarily transmitted via aerosol, respiratory droplets, or contact with skin lesions.[4,5] Recent outbreaks of clade IIb mpox virus have shown genital and rectal lesions as a frequent clinical manifestation, a pattern not previously prevalent among other orthopoxviruses that infect humans.[4,5] Due to this distinctive feature, we assessed whether the JYNNEOS vaccine also induced mucosal antibodies. We measured virus-reactive antibody responses in saliva and rectal swabs of individuals recently vaccinated with JYNNEOS. Saliva samples were analysed between 2 months and 7 months after vaccination, whereas rectal swab samples were collected at least 6 months after the second dose of JYNNEOS. IgA titres against a mix of vaccinia virus and mpox virus antigens were normalised to total IgA levels to estimate antibody avidity (appendix p 8). Although virus-reactive antibodies were detectable in the saliva of participants vaccinated with JYNNEOS, no antibodies were detected in rectal swabs.

We assessed plasma neutralisation activity against vaccinia virus, cowpox virus, and clade IIb mpox virus. All vaccination regimens effectively induced neutralising titres against vaccinia virus. 16 (40%) of 40 participants who received one dose of Dryvax, 48 (77%) of 62 participants who received two doses of JYNNEOS, and 29 (100%) of 29 participants who received a combination of three doses (Dryvax followed by JYNNEOS booster) showed neutralisation capacity against vaccinia virus compared with non-vaccinated controls (figure 2A). The most significant increase in neutralisation activity was seen in individuals vaccinated with Dryvax and recently boosted with JYNNEOS (figure 2A; appendix p 9). Neutralising activity against vaccinia virus directly correlates with anti-L1, anti-A33, and anti-B5 antibody titres (appendix p 9). Cross-protective neutralisation against related poxviruses, such as cowpox virus and mpox virus, was scarce and correlated with antigenic similarity to vaccinia virus (figure 2A, B). Neutralisation activity against cowpox virus (more antigenically related to vaccinia virus) was detected in all vaccinated groups, but neutralisation against mpox virus (more antigenically distant) was only observed in the boosted (Dryvax plus JYNNEOS) participants, suggesting enhanced cross-reactivity with additional boosters (figure 2A, B). Only individuals with high neutralisation titres for vaccinia virus (>2·5) showed neutralisation titres greater than 1·5 (exceeding the 1:30 dilution) to mpox virus (appendix p 9). Neutralisation activity against mpox virus was highest in cohort 4, consistent with infection-induced responses (figure 2A). A combination of monoclonal antibodies targeting both mature virions and extracellular virions was used as an experimental positive control and induced full virus neutralisation against both vaccinia virus and mpox virus

	Samples (n=378)	Mean (SD)	Participants (n=271)	p value
(Continued from previous page)				
Dose 1 30–60 days	14/61 (23%)	··	··	··
Dose 2 7–10 days	13/61 (21%)	··	··	··
Dose 2 30–60 days	14/61 (23%)	··	··	··
Dose 2 ≥180 days	4/61 (7%)	··	··	··
Vaccination interval, days	··	42·38 (8·90)	··	··
Regular (<40)	6/61 (10%)	29·20 (1·10)	5/29 (17%)	··
Extended (≥41)	50/61 (82%)	45·84 (6·33)	19/29 (66%)	··
No data	5/61 (8%)	··	5/29 (17%)	··
Mucosal samples				
Saliva	4/61 (7%)	··	4/29 (14%)	··
Rectal swab	3/61 (5%)	··	3/29 (10%)	··
Mpox convalescent cohort				
Age, years	··	33·72 (10·26)	··	··
20–29	19/68 (28%)	24·22 (2·54)	19/68 (28%)	<0·0001
30–39	30/68 (44%)	34·09 (2·70)	30/68 (44%)	··
40–49	13/68 (19%)	43·33 (2·08)	13/68 (19%)	··
50–59	5/68 (7%)	56·00 (0)	5/68 (7%)	··
60–69	1/68 (1%)	61·00 (0)	1/68 (1%)	··
Sex				
Male	66/68 (97%)	··	66/68 (97%)	··
Female	2/68 (3%)	··	2/68 (3%)	··
Dryvax vaccinated				
Yes	2/68 (3%)	··	2/68 (3%)	··
No	22/68 (32%)	··	22/68 (32%)	··
Unsure	44/68 (65%)	··	44/68 (65%)	··
Immune status				
HIV-positive	56/68 (82%)	··	56/68 (82%)	··
Transplant recipient	1/68 (1%)	··	1/68 (1%)	··
Immunocompetent	11/68 (16%)	··	11/68 (16%)	··
Hospitalisation				
Yes	3/68 (4%)	··	3/68 (4%)	··
No	22/68 (32%)	··	22/68 (32%)	··
No data	43/68 (63%)	··	43/68 (63%)	··
Days post-symptom onset				
0–30	46/68 (68%)	10·34 (7·43)	46/68 (8%)	··
31–60	4/68 (6%)	38·00 (8·04)	4/68 (6%)	··
≥61	1/68 (1%)	80·00 (0·00)	1/68 (1%)	··
No data	17/68 (25%)	0·00 (0·00)	17/68 (25%)	··
Controls (non-vaccinated)				
Age, years				
20–29	19/25 (76%)	··	19/25 (76%)	<0·0001
30–39	4/25 (16%)	··	4/25 (16%)	··
40–49	2/25 (8%)	··	2/25 (8%)	··
Sex				
Male	8/25 (32%)	··	8/25 (32%)	··
Female	16/25 (64%)	··	16/25 (64%)	··
No data	1/25 (4%)	··	1/25 (4%)	··
Mucosal samples				
Saliva	3/25 (12%)	··	3/25 (12%)	··
Rectal swab	0	··	0	··

Data are n/N (%), unless otherwise indicated. p values were assessed using one-way ANOVA within each cohort, α=0·05. *The baseline for the Dryvax plus JYNNEOS cohort represents the total Dryvax extended cohort.

Table: Cohort demographics



(Figure 2 continues on next page)

(appendix p 9). Neutralising antibodies against vaccinia virus and cowpox virus were also observed in infected patients, which might reflect previous smallpox vaccination. In a group of non-previously vaccinated participants infected with mpox virus IIb, a similar pattern of antibody cross-reactivity was observed: neutralising antibody levels correlated with antigenic similarity (figure 2C). Consistent with the systemic antibody level analysis, the peaks of neutralisation titres for vaccinia virus and cowpox virus were observed after the second JYNNEOS dose in cohorts 2 and 3 (figure 2D, F). The number of vaccine doses affected the magnitude, breadth, and kinetics of antibody responses against cross-reactive viruses (figure 2D–G). Booster doses promoted prolonged cross-reactive responses. Specifically, in orthopoxvirus naive individuals, two doses of JYNNEOS induced high neutralising titres against vaccinia virus and cowpox virus, but cowpox virus titres declined to baseline within 6–11 months. No neutralisation titres against mpox virus were observed (figure 2D, E; appendix p 9). In individuals previously vaccinated with Dryvax and then given two doses of JYNNEOS (totaling three doses), neutralisation of vaccinia virus, cowpox virus, and mpox virus was observed. Although neutralising antibodies against vaccinia virus and cowpox virus remained stable after 1 year, mpox virus-specific titres decreased to baseline levels within 6–11 months (figure 2F, G; appendix p 9). No significant differences in neutralising antibody titres against vaccinia virus or mpox virus were observed when the JYNNEOS vaccine interval was extended (>41 days; appendix p 9). Our data indicate that both first-generation and third-generation smallpox vaccines induce potent neutralising responses against vaccinia virus. However, despite the detection of high levels of anti-mpox virus-specific antibodies in the plasma, neutralisation activity directly correlated with viral antigenic distance, with a poor neutralising response observed for mpox virus in contrast to cowpox virus. Additionally, the number of vaccine boosters affects not only the magnitude but also the breadth and kinetics of the response,

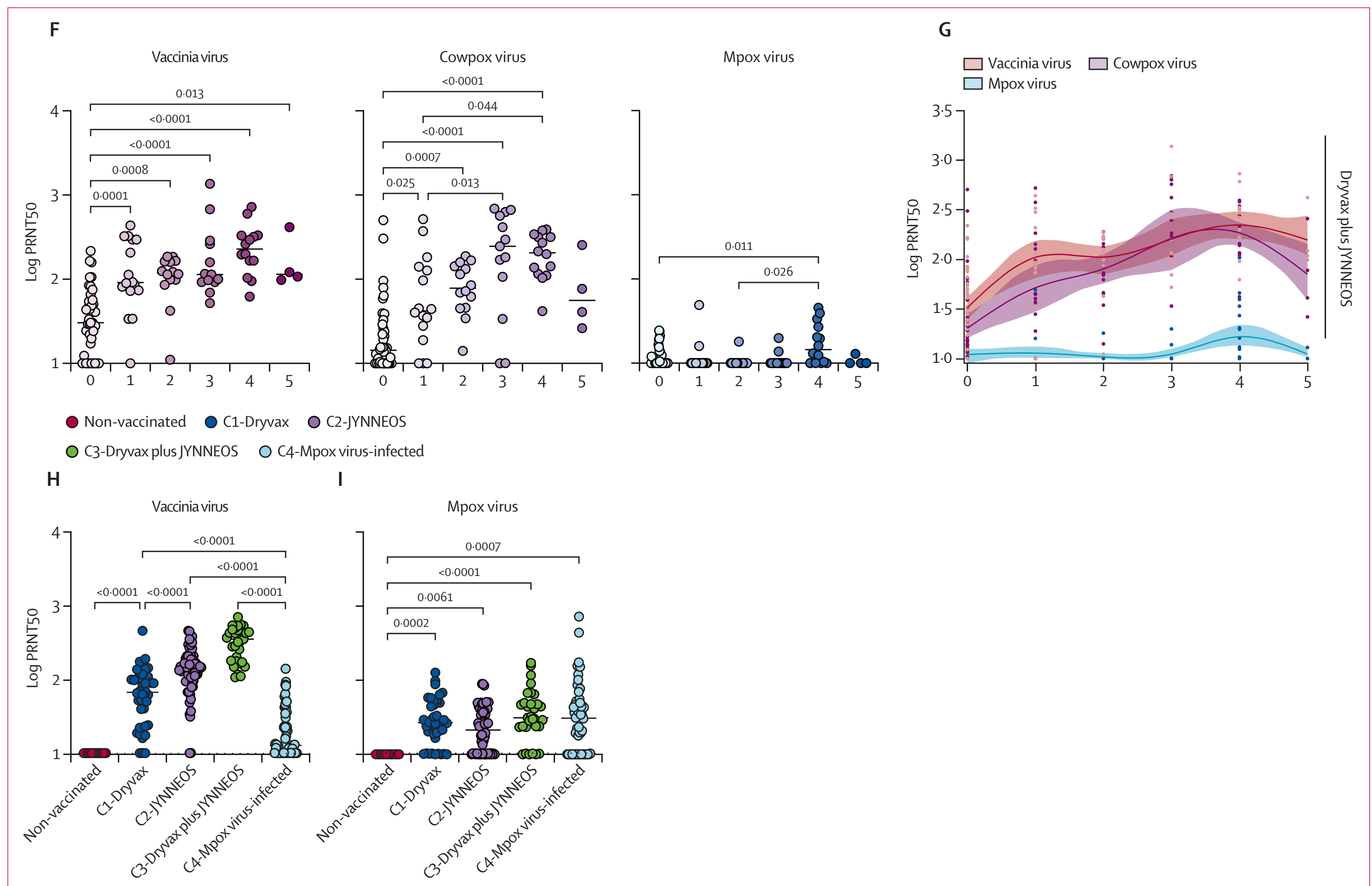


Figure 2: **Dynamics of plasma neutralisation cross-reactive responses against orthopoxviruses after vaccination or infection**

(A) Plasma direct neutralisation titres against vaccinia virus, cowpox virus, and mpox virus across fully vaccinated individuals, patients infected with mpox virus, and non-vaccinated controls. Individual points represent individual participants. Values are displayed as the $\log_{10}$ of 50% plaque reduction neutralisation titres (Log PRNT50). (B) Schematic figure illustrating antigenic similarity from vaccinia virus across orthopoxviruses used within this study. Arrows indicate antigenic distance. Full genomic similarity is indicated outside parentheses, and the percentage of genomic similarity across the eight immunogenic proteins, which are the main targets for neutralisation, is specified within the parentheses. Figure created with BioRender.com. (C) Plasma direct neutralisation capacity against vaccinia virus, cowpox virus, and mpox virus in non-vaccinated patients infected with mpox virus. Each point represents a single patient. (D–G) Plasma direct neutralisation capacity against vaccinia virus, cowpox virus, and mpox virus over time after JYNNEOS vaccination. Individual points represent individual participants. (D) Neutralisation titres over time in naive, non-vaccinated individuals after the JYNNEOS regimen. (E) LOWESS regression comparisons of plasma neutralisation capacity against each orthopoxvirus over time in naive, non-vaccinated individuals, after the JYNNEOS regimen. Regression lines are shown as pink (vaccina virus), purple (cowpox virus), and light blue (mpox virus); shading represents 95% CIs. (F) Neutralisation titres over time after the JYNNEOS regimen, in individuals previously vaccinated with Dryvax. (G) Locally weighted scatterplot smoothing regression comparisons of plasma neutralisation capacity against each orthopoxvirus over time following the JYNNEOS regimen, in individuals previously vaccinated with Dryvax. Regression lines are shown as pink (vaccinia virus), purple (cowpox virus), and light blue (mpox virus); shading represents 95% CIs. Complement-mediated neutralisation titres were assessed against fresh viral stocks of vaccinia virus (H) and mpox virus (I) across fully vaccinated individuals, individuals infected with mpox virus, and non-vaccinated controls. Individual points represent individual participants. In parts E and G, lines indicate neutralisation dynamics across vaccine regimens. In parts A, D, F, H, and I, horizontal bars represent mean (SD); cutoffs were defined by mean plus 2 SDs from non-vaccinated controls and are represented by dashed lines. For the x-axis labels in parts D and F, 0 indicates baseline, 1 indicates 7 days after first vaccine dose, 2 indicates 30–60 days after first vaccine dose, 3 indicated 7 days after second vaccine dose, 4 indicates 30–60 days after second vaccine dose, and 5 indicates 210–335 days after first vaccine dose.

with individuals who received three vaccination doses displaying the most robust, diverse, and prolonged cross-reactive responses.

To better understand the observed variations in antibody neutralisation titres, we investigated the genetic differences between vaccinia virus immunogenic proteins and their orthologues in cowpox virus and mpox virus isolates. Sequence analysis of five vaccinia virus immunogenic proteins (B5, A33, A27, L1, and D8) revealed overall high genetic identity (94–100%), with cowpox virus antigens showing higher sequence similarity to vaccinia virus proteins than mpox virus orthologues (appendix p 11). We identified all residues that were identical for cowpox virus and vaccinia virus but different for mpox virus (appendix p 11). Structural analysis indicates that most homologue mismatches identified in sequence alignment do not overlap with neutralisation epitope regions. However, certain residues in the mpox virus sequence, such as Gln117Lys and Leu118Ser in *A35R* and Thr146Met, Leu66Ioe, and Ser65Thr in *E8L* (appendix p 11), are adjacent to these sites and might affect the binding of neutralising antibodies.

The highest levels of virus-reactive antibodies in our vaccinee cohorts target extracellular virion antigens B5 and

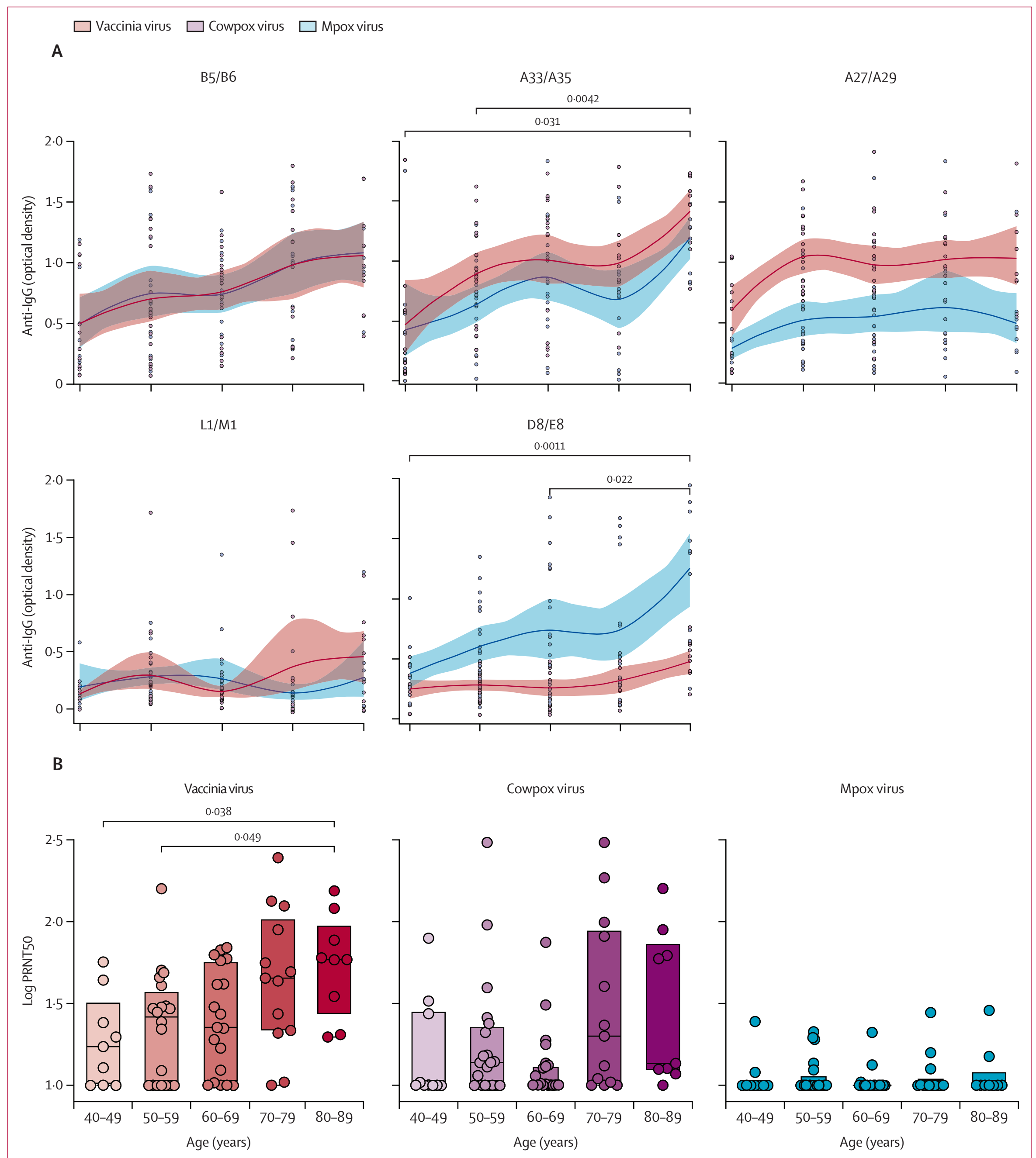
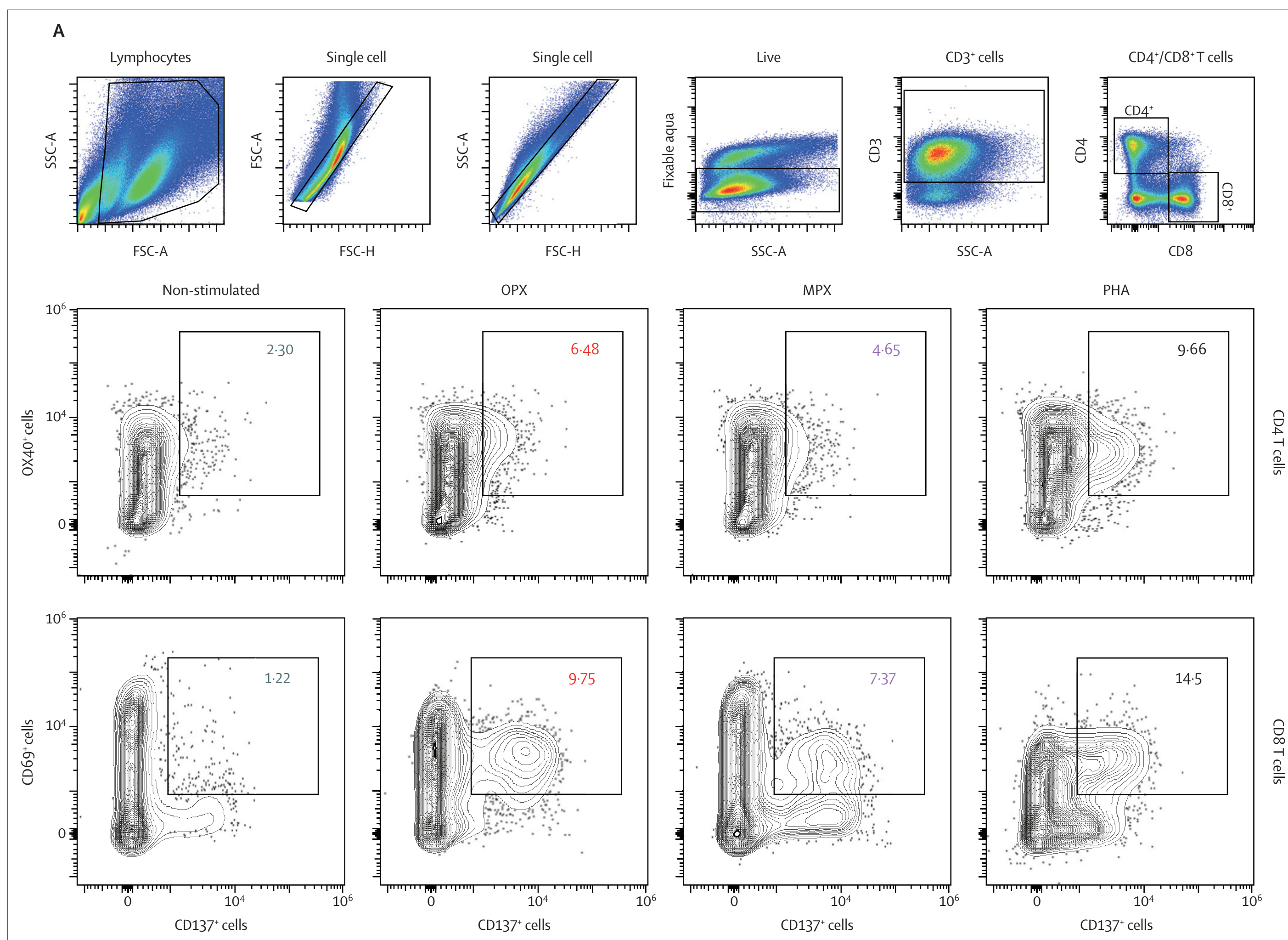


Figure 3: **Long-term cross-reactive humoral memory response after Dryvax**
(A) Plasma reactivity to vaccinia virus and mpox virus clade Ia proteins (B5/B6, A33/A35, A27/A29, L1/M1, and D8/E8) in individuals vaccinated with Dryvax across age groups. Regression lines indicate plasma IgG concentrations for anti-vaccinia virus antigens (pink) or anti-mpox virus antigens (blue); shading represents 95% CIs. (B) Plasma neutralisation titres against authentic vaccinia virus (VACV-VV.NP-S), cowpox virus (CWPXV-Brighton Red), and mokeypox virus (MPXV-hMPXV/USA/MA001/2022 [lineage B.1, clade IIb]), across age groups. Individual points represent individual patients.

A33 (figure 1C). Previous studies indicated that direct neutralisation of vaccinia virus extracellular virion by anti-A33 or anti-B5 antibodies does not occur even at high antibody concentrations but can be enhanced by complement.[17,18] To explore the role of antibody Fc effector functions in cross-protection, neutralisation assays were done with higher levels of complement. Although complement did not significantly enhance vaccinia virus neutralisation, it improved neutralisation capacity for mpox virus clade IIb (figure 2H, I). Representative viral plaques and



(Figure 4 continues on next page)

controls are shown in the appendix (p 12). These findings indicate that antibody-mediated protection conferred by smallpox vaccines against mpox virus is predominantly mediated by isotype-dependent effector functions, partly overcoming the limitations imposed by antigenic distance for cross-reactive responses.

Smallpox vaccines provide a valuable model to study immune response longevity, as typically individuals are not exposed repeatedly. However, the mechanisms underlying memory maintenance, particularly the persistence of cross-reactive memory B cells during an immunodominant primary response, remain unclear. We next assessed immune memory in individuals vaccinated decades ago with Dryvax vaccine (cohort 1) by expanding our initial cohort to include participants aged 60–89 years. Given that all participants from cohort 1 reported receiving a single smallpox vaccine during childhood (ages 0–10 years), and considering the self-reported nature of vaccination dates, we chose to stratify participants by age decades rather than by time since vaccination. The persistence of humoral responses over time was evaluated cross-sectionally using ELISA and neutralisation assays. Anti-vaccinia virus and anti-mpox virus IgG concentrations remained detectable for several decades after vaccination, with mpox virus cross-reactive responses following similar kinetics as vaccinia virus antigens (figure 3A). Neutralising activity against vaccinia virus, cowpox virus, and mpox virus was also evaluated over time. Neutralising titres against vaccinia virus gradually increased across age groups, suggesting an improvement in antibody affinity over time (figure 3B). Conversely, neutralising titres against cowpox virus and mpox virus did not show the same dynamics and maintained stable levels (figure 3B). These findings indicate that despite similar antibody dynamics, an increase in neutralisation titres is specific to the primary response but does not extend for cross-reactive antigens. Cross-reactive responses are less durable. Our analysis also indicates an unexpected improvement in the antibody neutralisation capacity that overcomes immune

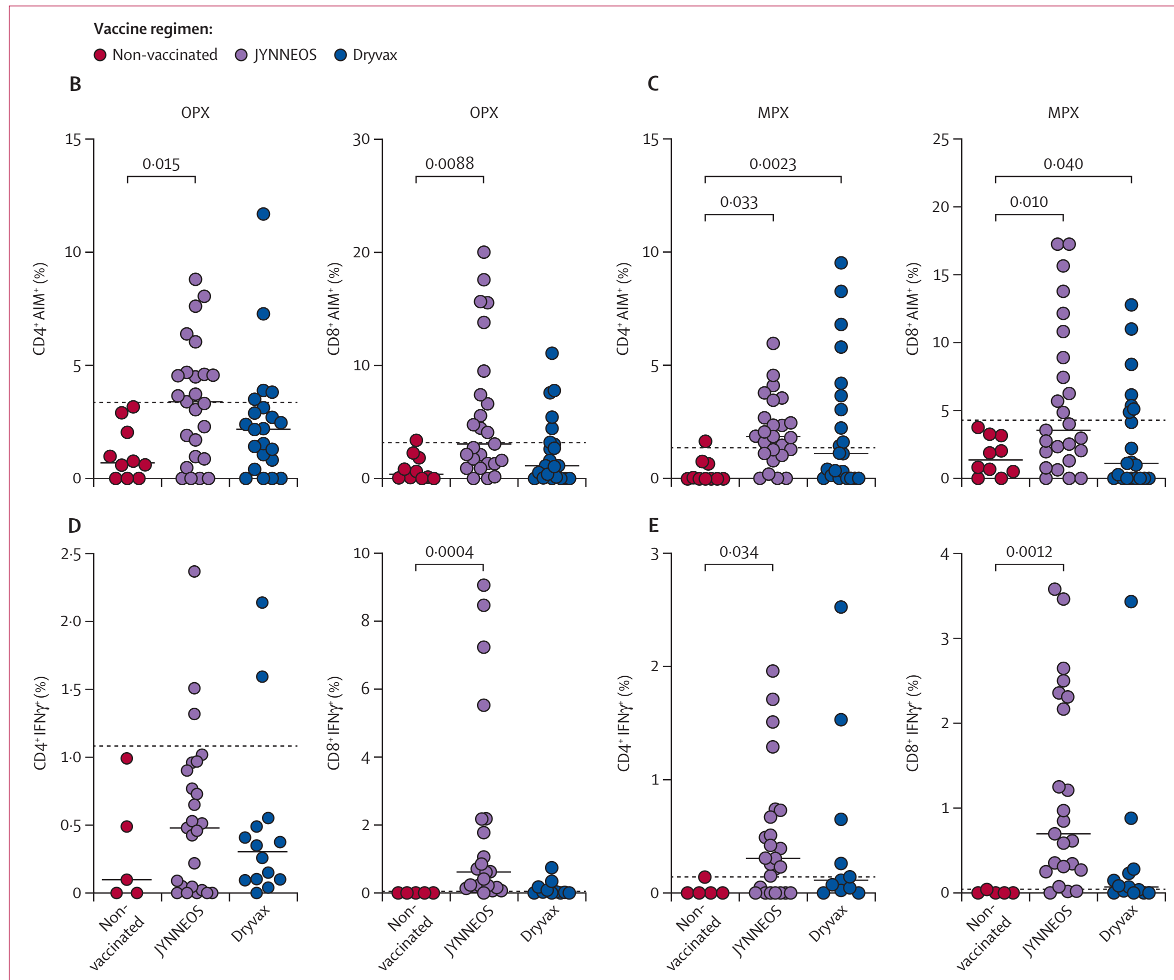


Figure 4: **Cross-reactive T-cell responses after first-generation or third-generation (or both) poxvirus vaccines**

(A) Gating strategy and representative dot plots showing the proportion of double-positive cells: OX40+ CD137+ (AIM+) CD4+ T cells (top six panels) and CD69+ CD137+ (AIM+) CD8+ T cells (bottom eight panels). (B, C) Surface marker analysis of AIM+ CD4+ T cells and AIM+ CD8+ T cells in individuals recently fully vaccinated with JYNNEOS compared with those vaccinated with Dryvax long ago. (B) Analysis of OPX-reactive AIM+ T cells. (C) Analysis of MPX-reactive AIM+ T cells. (D, E) ICS analysis of AIM+ CD4+ T cells and AIM+ CD8+ T-cell activation in individuals recently fully vaccinated with JYNNEOS compared with those vaccinated with Dryvax long ago. (D) ICS analysis of OPX-reactive AIM+ T cells. (E) ICS analysis of MPX-reactive AIM+ T cells. In parts B–E, each dot represents a single individual, and the indicated percentage values have been adjusted by subtracting the background stimulation observed in non-stimulated cell controls. Horizontal bars represent mean (SD). Cutoffs were defined by mean plus 2 SDs from non-vaccinated controls and are represented by dashed lines. AIM=activation-induced marker. FSC-A=forward scatter area. FSC-H=forward scatter height. ICS=intracellular cytokine staining. MPX=mpox peptide pool. OPX=orthopoxvirus peptide pool. PHA=phytohaemagglutinin. SSC-A=side scatter area.

senescence, with individuals aged 80 years showing the most robust humoral response against vaccinia virus.

Previous research on vaccinia virus has shown that, although antibody responses are crucial for disease prevention, T-cell responses are important for controlling and terminating poxvirus infections.[19,20] To understand the full scope of cross-protection conferred by smallpox vaccines, we evaluated T-cell responses for their ability to recognise orthopoxvirus peptide pools or a specific mpox peptide pool, using activation-induced marker assays and intracellular cytokine staining, as previously described.[13] To detect low-frequency peptide-specific T cells, PBMCs from vaccinated individuals were stimulated ex vivo with orthopoxvirus peptide pools or mpox peptide pools for 9 days. We observed that[21] JYNNEOS vaccination led to an increase in orthopoxvirus peptide pool-reactive CD4 and CD8 T cells, as evidenced by upregulation of activation markers, including OX-40, CD69, and CD137 (figure 4A). Furthermore, we found that both first-generation and third-generation vaccinia virus-based vaccines induce T-cell responses that cross-recognise mpox virus-derived epitopes (figure 4A). Such cross-reactivity was confirmed in individuals recently vaccinated with JYNNEOS and those vaccinated long ago with Dryvax, including those who received Dryvax vaccines more than 80 years ago (figure 4B, C). Both CD4 and CD8 T cells from recently vaccinated individuals produced IFN-γ in response to mpox virus peptide pools compared with non-vaccinated individuals (figure 4D, E). Although IFN-γ production was observed in some individuals vaccinated with Dryvax long ago, the levels were not significant

(figure 4D, E). To investigate the variability in T-cell responses among individuals vaccinated long ago, we stratified participants by age group. Analysis of Dryvax-vaccinated individuals indicates that T-cell responses are affected by immunosenescence and diminish with age. This finding was observed after stimulation with both orthopoxvirus and mpox virus peptide pools, and with the positive control (appendix p 12). Thus, our data show that smallpox vaccines effectively induce long-term T-cell responses to vaccinia virus antigens, and cross-reactive responses to mpox virus. However, in contrast to B-cell responses, T-cell responses decrease with age.

Discussion

In the wake of recent mpox outbreaks, this study provides essential insights into the cross-reactive immune responses elicited by smallpox vaccines. Due to the highly conserved nature of orthopoxviruses, smallpox vaccines are believed to be cross-protective against other orthopoxviruses, leading to the authorisation of both ACAM2000 and JYNNEOS to be used prophylactically against mpox infection. However, without thorough evaluation of cross-protection and memory responses elicited from smallpox vaccines, there are substantial gaps in understanding of the effectiveness and duration of these vaccines against related orthopoxvirus infections. We show that both first-generation and third-generation smallpox vaccines elicit cross-reactive antibody and T-cell responses, but humoral responses to mpox virus are restricted. The correlation between antigenic distance and direct neutralisation emphasises the challenge of achieving broad protection, which can be addressed through antibody effector functions. Cross-reactive T-cell responses persist for over 80 years after vaccination, unlike humoral responses, but are impacted by ageing. Booster doses improve both the breadth and durability of cross-reactive immune responses.

Cross-reactive immunity to mpox virus has been previously observed in mouse models and Dryvax-vaccinated individuals, with studies reporting vaccinia virus-reactive and mpox virus-neutralising antibodies.[22,23] We showed that neutralisation capacity is affected by antibody titres and antigenic distance. Although JYNNEOS boosting in Dryvax-vaccinated individuals only generated transient humoral responses (<11 months), multiple boosts led to long-lasting cross-reactive humoral responses.[24] Structural and antigen-binding analyses suggest that diminished mpox responses are probably due to reduced homology between vaccinia virus and the 2022 mpox strain.[25] This antigenic distance phenomenon, observed in other viruses, such as like influenza and SARS-CoV-2, highlights challenges in mounting cross-reactive responses when antigenic similarity is low.[26–28] Our findings suggest that boosting might improve antibody breadth either by increasing titres of cross-protective antibodies or through immunodominant epitope masking. These results provide a framework for designing vaccines that target conserved antigens for broad protection.

Given the high concentrations of systemic mpox-reactive antibodies but low titres of neutralising antibodies against mpox virus in our vaccine cohorts, we investigated additional antibody-mediated mechanisms of protection. Notably, antibodies against the extracellular virion form, specifically anti-B6/B6 and anti-A33/35, were the most abundant antibodies produced by the vaccinated participants in our cohorts. Previous studies indicate that direct neutralisation of vaccinia virus extracellular virion by anti-A33 or anti-B5 antibodies does not occur, even at high concentrations, but can be enhanced in the presence of complement.[17,18] Our findings indicate that complement enhances mpox virus neutralisation, underscoring the importance of Fc effector functions in partly overcoming the limitations imposed by antigenic distance. Recent studies in macaques using a novel mpox virus-derived mRNA vaccine showed broader protection against diverse orthopoxviruses, including mousepox, rabbitpox, and camelpox.[29] Although these findings need confirmation in humans, a striking difference between the mRNA vaccine and existing smallpox vaccines is potent induction of responses against the conserved M1/L1 protein—the most stable neutralisation target across orthopoxviruses. By contrast, Dryvax and JYNNEOS primarily induce antibody responses against the less conserved extracellular virion antigens B5 and A33, which mediate protection through Fc function. These insights expand our understanding of antibody-mediated protection against orthopoxvirus and highlight the crucial role of antibody effector functions for cross-protective responses.

Similar to previous reports, we observed detectable anti-vaccinia virus antibody responses in patients vaccinated up to 80 years ago.[9,30] Remarkably, stratifying participants by age revealed that neutralisation capacity against vaccinia virus increased over decades. This phenomenon might reflect unique mechanisms of sustained memory B-cell activation, warranting further investigation. Memory T-cell responses to both vaccinia virus and mpox virus persisted for up to 80 years after vaccination, showing the durability of cell-mediated immunity. However, cross-reactive T-cell responses were more robust in recently vaccinated individuals and diminished with ageing. Our study expands on previous findings by providing a comprehensive analysis of T-cell responses induced by first-generation and third-generation vaccines to both vaccinia virus and mpox virus. However, functional analysis to assess the protective role of T-cell responses in human donors will need to be further investigated.

This study has several limitations. Cohort heterogeneity, including differences in demographics and vaccine administration (Dryvax *vs* JYNNEOS), might affect comparability. The inability to differentiate between specific and cross-reactive antibodies limits insights into immune specificity. Additionally, antigenic distance analysis highlighted a relationship between mutations and neutralisation capacity, but the effects of individual mutations remain unclear. The activation induced marker assay for analysing T-cell

response was used on cells after 9-day in-vitro stimulation, which might have affected the expression of membrane markers and results in high background levels. Future studies should address these limitations to refine our understanding of cross-protective immune responses.

In conclusion, our study brings new insights into the comparative immune responses between diverse poxvirus vaccines and orthopoxvirus infections. Our findings underscore the importance of humoral and cellular immunity for cross-protection and provide valuable information for designing future orthopoxvirus vaccines and therapies.

Contributors

Conceived the study: LP, IY, SBO, and CL. Brazilian cohorts set up, sample collection, and processing: LC, GM, GMdA, TMC, GSL, LMH, and AMV. Portugal cohort set up, sample collection, and processing: JC, ARP, VA, DP, FM, and RC. US cohort set up and sample collection: LP, IY, AZ, LA-B, SBO, and CL. US cohort sample processing: VSM, YZ, and LL. Virus sequencing: MIB, CBFV, and NDG. Cell culture and virus expansion: YZ and LL. Experiments and data collection: JC, VSM, LP, YZ, and CL. Data analysis: JC, VSM, AMV, and CL. Virus sequence identity analysis: ZF and SC. T-cells peptide pool design: AG and AS. Writing—original draft: JC, VSM, ZF, and CL. Supervised and submitted the research for publication: SBO and CL. All authors reviewed and approved the manuscript for publication. Authors JC, VSM, LP, ZF, LC, YZ, RC, IY, AMV, SBO, and CL had access to the raw data and verified the underlying data reported in the manuscript.

Declaration of interests

AS is a consultant for Gritstone Bio, Flow Pharma, Moderna, AstraZeneca, Qiagen, Fortress, Gilead, Sanofi, Merck, RiverVest, MedaCorp, Turnstone, NA Vaccine Institute, Emervax, Gerson Lehrman Group, and Guggenheim. AG is a consultant for Sanofi and Pfizer. La Jolla Institute for Immunology LJI (AS and AG) has filed for patent protection for various aspects of T-cell epitope and vaccine design work. All other authors declare no competing interests.

Data sharing

The genome information and aligned consensus genomes for poxviruses used in this study are available on the National Center for Biotechnology Information (GenBank accession numbers of Copenhagen [Cop]-D8L, Cop-A27L, Cop-A33R, Cop-L1R, and Cop-B5R antigens of mpox 2022 USA strain, mpox Zaire-96-I-16 strain, and cowpox and their identity with corresponding antigens of Vaccinia virus WR strain are listed below). The mpox 2022 genome (GCA_025462475.1) and reference genomes of mpox Zaire strain (GCF_000857045.1), vaccinia virus WR strain (GCF_000860085.1), and cowpox (GCF_000839185.1) were used in the genome sequence alignment and analysis. Additional analyses of poxvirus antigen sequences and genomes are derived from the mpox 2022 strain (GCA_025462475.1), mpox Zaire strain (GCF_000857045.1), vaccinia virus WR strain (GCF_000860085.1), cowpox Brighton Red (GCF_000839185.1), vaccinia virus Copenhagen (GCA_006458465.1), smallpox-Bangladesh-1975 (GCA_006465665.1), modified vaccinia Ankara (MVA)-BN (GCA_023530715.1), MVA-ACAM3000 (GCA_023535515.1), MVA (GCA_023533565.1), MVA Ducapox (GCA_023532165.1), vaccinia NYCBH (Dryvax) ACAM2000 (GCA_023533825.1), Dryvax-3737 (GCA_023536375.1), vaccinia virus Lister-LC16m8 (GCA_023535895.1), vaccinia virus Tian Tan TT12 (GCA_023535555.1), and vaccinia Ankara (GCA_006457925.1) genomes. Additional correspondence and requests for materials should be addressed to the corresponding author (carolina.lucas@yale.edu).

1. Vaccinia virus WR strain as sequence alignment references:
   a. Cop-D8L: QTC35383.1, 100%
   b. Cop-A27L: QTC35423.1, 100%
   c. Cop-A33R: QTC35435.1, 100%
   d. Cop-L1R: QTC35412.1, 100%
   e. Cop-B5R: QTC35539.1, 100%
2. Mpox 2022 USA strain:
   a. E8L, Cop-D8L: URK20542.1, 95%
   b. A29L, Cop-A27L: URK20577.1, 94%
   c. A35R, Cop-A33R: URK20584.1, 95%
   d. M1R, Cop-L1R: URK20517.1, 99%
   e. B6R, Cop-B5R: URK20605.1, 96%
3. Mpox Zaire-96-I-16 strain:
   a. E8L, Cop-D8L: AAL40563.1, 94%
   b. A29L, Cop-A27L: AAL40597.1, 94%
   c. A35R, Cop-A33R: AAL40603.1, 95%
   d. M1R, Cop-L1R: AAL40538.1, 99%
   e. B6R, Cop-B5R: AAL40625.1, 96%
4. Cowpox:
   a. CPXV125, Cop-D8L: ADZ30306.1, 98%
   b. CPXV162, Cop-A27L: AAP48882.1, 98%
   c. CPXV168, Cop-A33R: ADZ29703.1, 99%
   d. CPXV99, Cop-L1R: ADZ24097.1, 100%
   e. CPXV199, Cop-B5R: ADZ29304.1, 97%

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