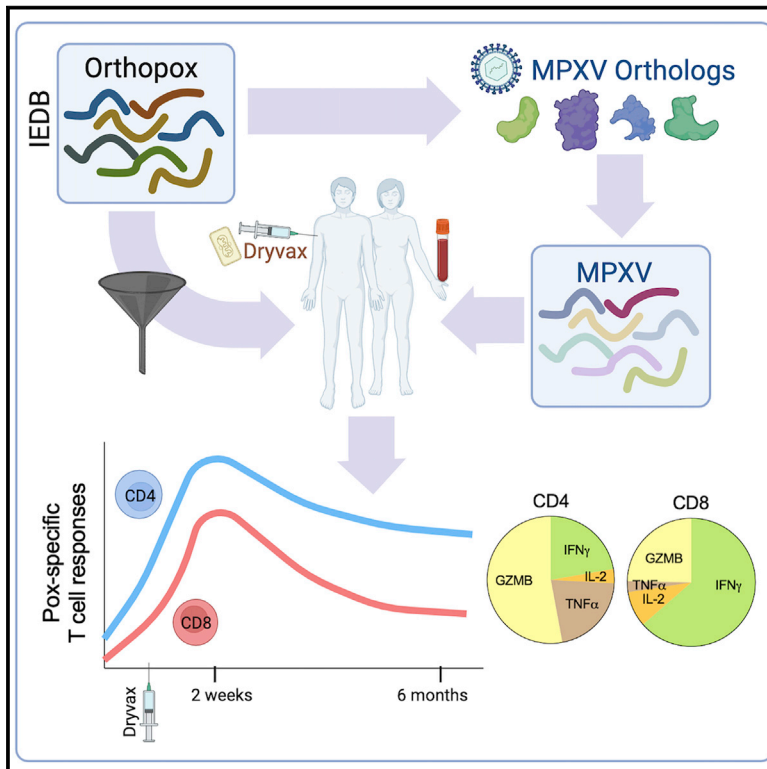# Cell Host & Microbe

Defining antigen targets to dissect vaccinia virus and monkeypox virus-specific T cell responses in humans

Graphical abstract

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In brief

Grifoni et al. developed orthopox- and monkeypox-specific epitope pools to measure monkeypox T cell responses in natural infection and vaccination. The pools were validated by detection of memory T cell responses in PBMCs from Dryvax vaccinees. A majority of the Dryvax-vaccinee CD4 responses were cytotoxic and produced granzyme B.

Highlights

- OPXV epitope pools detect CD4 and CD8 T cell responses in 100% and 79% of Dryvax vaccinees, respectively

- Predicted MPXV pools detect CD4 and CD8 T cells in 100% and 53% of Dryvax vaccinees, respectively

- Vaccination yields memory T cells, with CD4 response more persistent than CD8 response

- Orthopox-specific CD4 response is multifunctional, and 50% of it is cytotoxic

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Short Article

Defining antigen targets to dissect vaccinia virus and monkeypox virus-specific T cell responses in humans

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SUMMARY

The monkeypox virus (MPXV) outbreak confirmed in May 2022 in non-endemic countries is raising concern about the pandemic potential of novel orthopoxviruses. Little is known regarding MPXV immunity in the context of MPXV infection or vaccination with vaccinia-based vaccines (VACV). As with vaccinia, T cells are likely to provide an important contribution to overall immunity to MPXV. Here, we leveraged the epitope information available in the Immune Epitope Database (IEDB) on VACV to predict potential MPXV targets recognized by CD4+ and CD8+ T cell responses. We found a high degree of conservation between VACV epitopes and MPXV and defined T cell immunodominant targets. These analyses enabled the design of peptide pools able to experimentally detect VACV-specific T cell responses and MPXV cross-reactive T cells in a cohort of vaccinated individuals. Our findings will facilitate the monitoring of cellular immunity following MPXV infection and vaccination.

INTRODUCTION

On August 24th, 2022, the World Health Organization (WHO) reported 41,664 confirmed cases of monkeypox virus (MPXV) infection and five deaths in non-endemic regions.[1] Although MPXV infections and outbreaks have been reported on the African continent in the past three decades, this current outbreak is unprecedented in size and scope; having spread globally to almost 100 countries, the vast majority of these countries have not historically reported MPXV cases, including European countries and the US.[2]

With the current outbreak, it is important to understand immunity against MPXV, but only a few studies have addressed immune responses to MPXV infections in humans.[3–5] First, little information is available on the quality and duration of immune responses to MPXV infection in humans. Second, little data on efficacy in humans are available for the MPXV vaccines based on

the vaccinia virus (VACV). Third, it is unknown to what extent human cellular immune responses induced by VACV vaccination are cross-reactive with MPXV. These knowledge gaps should be addressed in MPXV and also in the strain associated with the current outbreak.[6]

Information is available on immune responses and correlates of protection from VACV infection,[7–9] the virus utilized as a vaccine to protect from smallpox disease caused by variola virus (VARV) infection.[10] Several VACV studies demonstrate that antibody responses are crucial for disease prevention,[8,9] whereas T cell responses are important to control and terminate pox-virus infections.[11,12] Many studies describe T cell epitopes for Orthopoxviruses (OPXVs), and VACV in particular;[12–19] however, only two studies have investigated T cell responses against MPXV in humans.[20,21]

VACV was utilized under the brand name of Dryvax to eradicate smallpox in the 1980s,[22] but Dryvax-vaccinated individuals
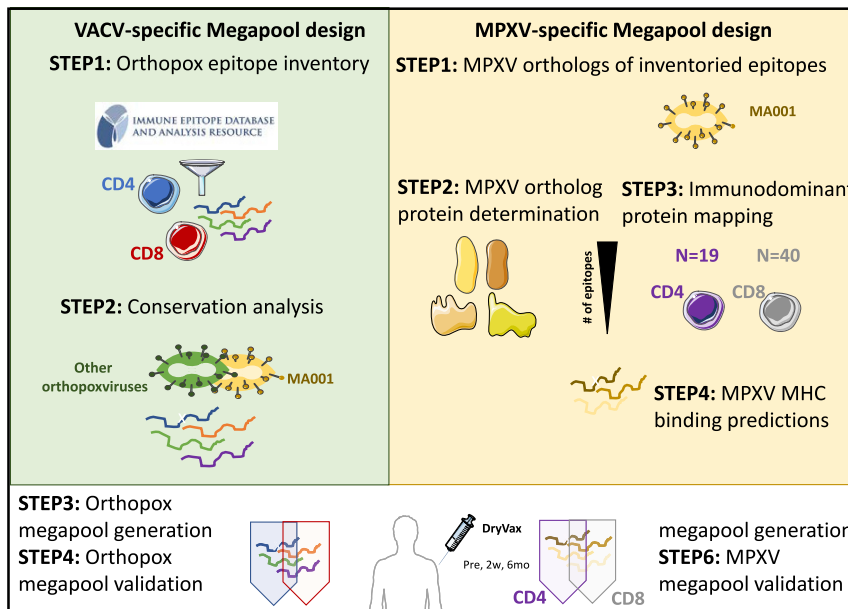
Figure 1. Schematic representation of peptide pools design

Pox viruses have relatively large genomes, encoding ~200 different open reading frames (ORFs),[32] with studies reporting broad immune responses to many ORFs.[19] To define MPs to measure T cell responses in MPXV vaccination and infection, we inventoried experimentally defined epitopes described in the literature and curated by IEDB, as of May 2022 (step 1, Figure 1A). The analysis identified 318 CD4$^+$ and 659 CD8$^+$ T cell epitopes derived from OPXVs (Table S1). The vast majority of epitopes (88%) have been described in the context of VACV, of which 78% of CD4$^+$ epitopes and 36% of CD8$^+$ epitopes were associated with responses in humans (Table S1).

Based on these data, we developed two MPs: OPX-CD8-E and OPX-CD4-E. For OPX-CD8-E, we selected the 238 CD8$^+$ epitopes recognized in humans, whereas for OPX-CD4-E, we included all 318 CD4$^+$ epitopes recognized in any species, based on the high degree of overlap between binding repertoires of MHC class II.[42] For the CD4$^+$ epitopes, we performed a clustering analysis to create epitope regions of up to 22 residues that encompass nested or overlapping epitopes. Accordingly, a set of 300 CD4$^+$ epitopes was generated and is listed in Table S1 along with the CD8$^+$ epitopes.

The MPXV and VACV viruses have been reported[32] to share a high degree of sequence homology and conservation, suggesting that Orthopox-specific T cell epitopes may be conserved in MPX.[6] We ascertained whether each epitope was conserved in the MPXV isolate MA001 (step 2, Figure 1A).

The results indicate that both CD4$^+$ and CD8$^+$ epitopes are highly conserved with 94% and 82%, respectively, being 100% conserved in MPXV (Table S1), with high conservation (range 74%–96%) irrespective of the viral species in which the epitopes were originally defined. The results indicated that most previously defined Orthopox epitopes were highly conserved and could be used to generate MPs as a potential reagent (step 3, Figure 1A).

T cell immune responses after Dryvax vaccination are detected by Orthopox MPs

We evaluated T cell responses for their capacity to recognize the Orthopox MPs, using peripheral blood mononuclear cells (PBMCs) in cohorts of Dryvax-vaccinated and non-vaccinated subjects (step 4, Figure 1A). Cohort characteristics are provided in Table S2 and STAR Methods. To measure the T cell responses to OPX-CD8-E and OPX-CD4-E, we combined activation-induced marker (AIM) assays with cytokine intracellular staining (ICS) (Figure S1A). For CD4$^+$ T cells, the highest responses were observed in recently vaccinated subjects. The magnitude of T cell reactivity was comparable pre-vaccination and 5–7 months post-vaccination (geometric mean [GM] ± geometric standard deviation

with impaired cellular immunity were deficient in VACV control[23,24] and risked severe reactions and safety issues.[25] Dryvax and Acambis 2000 (the related attenuated vaccine) vaccination was largely discontinued after 2001 and replaced by the modified vaccinia Ankara (MVA) virus (under the brand name JYNNEOS), which has a superior safety profile[26] and induces similar levels of antibody responses.[27]

Studies in non-human primate models using VACV vaccination to prevent MPXV infection show efficacy in preventing infection and/or attenuating disease severity.[5,28–30] Data from human studies are limited to an observational study demonstrating 85% efficacy in preventing MPXV disease in subjects vaccinated with Dryvax.[31] The JYNNEOS vaccine is approved for use to prevent MPXV infection/disease based on serological responses, but no data are available addressing clinical efficacy in humans. An additional knowledge gap is the degree of T cell epitope conservation elicited by VACV vaccination for MPXV infection. MPXV shares 90% overall sequence homology with VACV,[32] suggesting that VACV-induced T cell responses might be largely cross-reactive with MPXV epitopes. VACV epitopes are largely conserved in VARV,[33] suggesting a similar strategy can be applied to other OPXVs. Here, we used the Immune Epitope Database and Analysis Resource (IEDB)[34] and Virus Pathogen Resource (ViPR)[35] to compile known OPXV T cell epitopes to determine protein conservation and assess immunodominance.

We previously showed that large pools of peptides (megapools [MPs])[36] can be used to measure CD4$^+$ and CD8$^+$ T cell responses against a number of allergens, as well as bacterial and viral targets.[37–41] Here, we develop and validate pools of previously defined epitopes to assess T cell responses to VACV and MPXV.

RESULTS

Orthopox T cell epitopes curated in IEDB and conservation of Orthopox epitopes within MPX
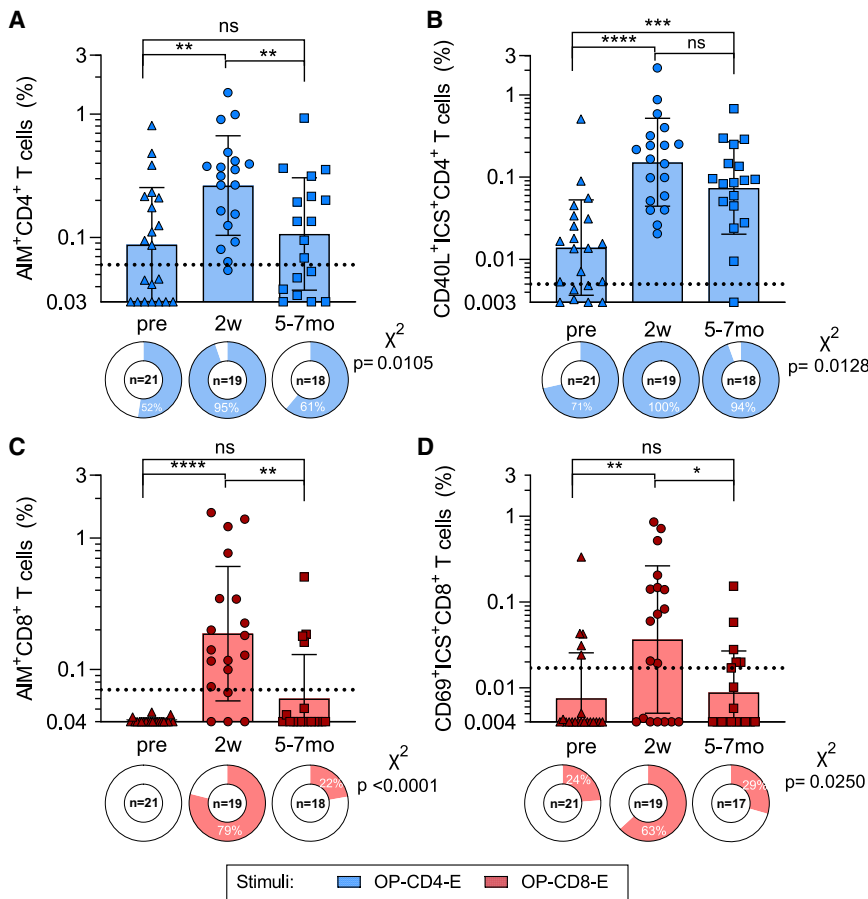
Figure 2. T cell responses against Orthopox MPs after vaccinia vaccination; refers to Figures S1 and S2 and Tables S1–S4

PBMCs from each time point were tested in AIM/ICS assays with experimentally defined OPXV MPs, OP-CD4-E (blue), and OP-CD8-E (red).

(A and B) OP-CD4-E-specific CD4$^+$ T cell reactivity is shown for (A) AIM and (B) ICS.

(C and D) OP-CD8-E specific CD8$^+$ T cell reactivity is shown for (C) AIM and (D) ICS. The y axis of each bar graph starts at the limit of detection (LOD), and the limit of sensitivity (LOS) is indicated with a dotted line. Columns represent the geometric mean and error bars indicate the geometric standard deviation. Pie charts below each bar indicate the frequency of positive responders. Mann-Whitney t test was applied to each graph, and p values symbols are shown when significant. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. X^2 test was applied to the frequencies of positivity, and p values are listed on the right of each graph.

post-vaccination samples reverting to a magnitude similar to pre-vaccination (GM ± GSD; pre = 0.04 ± 1.04, 2 weeks = 0.19 ± 3.24, and 5–7 months = 0.06 ± 2.15; p < 0.0001 Kruskal-Wallis test; in Figure 2C). The frequency of positive CD8$^+$ T cell responses increased to 79% 2 weeks post-vaccination and decreased to 22% at the 5–7 months' time point (χ^2 p < 0.0001). Similar results were observed in the ICS assay, where IFNγ, GZMB, TNF-α, and IL-2 secreting CD69$^+$CD8$^+$ T cells were quantified (GM ± GSD; pre = 0.01 ± 3.39, 2 weeks = 0.04 ± 7.22, and 5–7 months = 0.01 ± 3.06; p = 0.01 Kruskal-Wallis test, χ^2 p = 0.025; Figure 2D). CD8$^+$ T cell functionality was also assessed based on IFNγ, TNF-α, IL-2, and/or GZMB expression and showed a prevalence of IFNγ followed by IL-2 and GZMB (Figures S2C–S2F).

Definition of immunodominant ORFs and prediction of MPX T cell epitopes

We next wanted to define MPs based on MPXV sequences that would be more suited to characterize responses in MPXV infection. We started with the same list of Orthopox epitopes (Table S1) and identified corresponding conserved sequences in MPXV (step 1, Figure 1B) with sequence identities of ≥70% for class II and ≥80% for class I epitopes. By mapping those epitopes to the corresponding MPXV ortholog proteins (step 2, Figure 1B), we derived a set of MPXV proteins that is potentially immunodominant (see Table S3). Accordingly, we identified 19 antigens for CD4$^+$ and 40 for CD8$^+$ (step 3, Figure 1B). The 19 MPXV CD4$^+$ ORFs accounted for 67% of the homologous IEDB Orthopox CD4$^+$ epitopes, and the 40 CD8$^+$ ORFs accounted for 61% of the corresponding IEDB CD8$^+$ epitopes (Figure 3). Overall, 48 proteins were immunodominant, corresponding to 25% of the MPXV proteome. T cell immunodominant targets identified were mostly early transcribed proteins.[43]

[GSD]; pre = 0.09 ± 2.90, 2 weeks = 0.26 ± 2.53, and 5–7 months = 0.11 ± 2.86; p = 0.004 Kruskal-Wallis test; Figure 2A). The frequency of CD4$^+$ T cell responders increased from 52% to 95% 2 weeks post-vaccination with a decline to 61% 5–7 months post-vaccination (χ^2 p = 0.01).

Similar findings were observed by ICS, although CD4$^+$ T cell responses showed a more modest and insignificant decline 5–7 months post-vaccination; responses post-vaccination were significantly higher in magnitude and frequency than in pre-vaccination (GM ± GSD; pre = 0.01 ± 3.80, 2 weeks = 0.15 ± 3.42, and 5–7 months = 0.07 ± 3.64; p < 0.0001 Kruskal-Wallis test, χ^2 p = 0.0128; Figure 2B). Unexposed subjects did not yield appreciable responses (Figures S1B and S1C). The ICS assays demonstrated that CD40L$^+$CD4$^+$ T cell responses were Th1 or cytotoxic responses, encompassing mostly granzyme B (GZMB) and IFNγ, followed by TNF-α and IL-2 (Figure S2). Significant increases in TNF-α and IL-2 and a decrease in GZMB production were observed at 5–7 months post-vaccination, as compared with 2 weeks (TNF-α: 2 weeks = 18% and 5–7 months = 33%, p = 0.0088; IL-2: 2 weeks = 3% and 5–7 months = 13%, p = 0.0059; GZMB: 2 weeks = 53% and 5–7 months = 35%, p = 0.0292). A prevalence of CD40L$^+$IFNγ$^+$GZMB$^+$ population followed by CD40L$^+$GZMB$^+$, CD40L$^+$IFNγ$^+$, and CD40L$^+$TNF-α$^+$ populations were observed (Figures S2A and S2E).

In CD8$^+$ T cells in AIM assays, the highest responses were observed in recently vaccinated subjects, with the 5–7 months
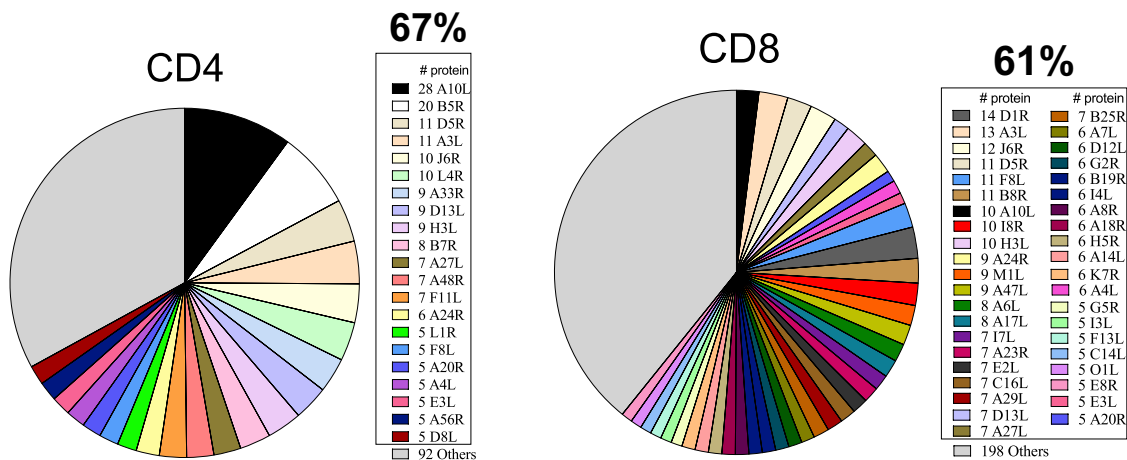
Figure 3. Orthopox protein immunodominance; refers to Table S1

The IEDB was mined for experimentally defined epitopes derived from Orthopoxviruses. A total of 47 different antigens were identified as the protein source for defined epitopes, corresponding to 19 and 39 antigens associated with CD4$^+$ or CD8$^+$ epitopes, respectively. The pie charts represent the total proteins recognized by CD4$^+$ and CD8$^+$ as defined by the IEDB and listed in Table S1. The most immunodominant antigens are listed in the figure legend, and the remaining antigens ("others") are colored in gray.

Several targets, such as A10L, A3L, D5R, A26L/A30L, H3L, J6R, A27L, D13L, A24R, A4L, and F8L, are of particular interest, since they were dominant for both CD4$^+$ and CD8$^+$ T cells. The study of immunodominant ORFs is also of particular relevance, since JYNNEOS vaccine is based on MVA, an attenuated VACV that lost 14% of the original genome,[44] retaining only 157 ORFs (Gen-Bank: U94848.1).[45] Importantly, all the ORFs considered as dominant for CD4$^+$, CD8$^+$, or both were conserved in the MVA/JYNNEOS sequences. This confirms the large breadth of immunogenic ORFs in OPXVs.[15] We predicted potential T cell epitopes from the MPXV orthologs of the 19 CD4$^+$ and 40 CD8$^+$ immunodominant antigens, using IEDB tools[46] (step 4, Figure 1B). For CD4$^+$, we predicted 276 promiscuous HLA class II binders (Table S3).[47] In parallel, we predicted 1,647 potential CD8$^+$ epitopes binding to a panel of common HLA class I alleles, as previously utilized in other viral systems[39,48] (step 5, Figure 1B; Tables S3–S4).

Assessment of CD4$^+$ and CD8$^+$ T cell cross-reactive responses able to recognize MPXV in smallpox-vaccinated individuals

We then evaluated whether VACV-induced T cell responses could cross-recognize the MPXV-derived, predicted epitope pools (Figures 4 and S2). By the AIM assay, the magnitude of CD4$^+$ T cell cross-reactivity peaked at 2 weeks post-vaccination and declined at 6–7 months, reaching comparable reactivity to pre-vaccination (GM ± GSD; pre = 0.06 ± 2.51, 2 weeks = 0.23 ± 2.91, and 5–7 months = 0.08 ± 2.63; p = 0.0005 Kruskal-Wallis test; Figure 4A). The frequency of CD4$^+$ T cell responders significantly increased from 43% to 84% 2 weeks post-vaccination, with a decline to 67% 5–7 months post-vaccination (χ^2 p = 0.024). Similar results were observed by ICS, although the post-vaccination decline was less pronounced and responses were still significantly higher than those observed pre-vaccination (GM ± GSD; pre = 0.011 ± 3.58, 2 weeks = 0.12 ± 4.67, and 5–7 months = 0.04 ± 4.13; p < 0.0001 Kruskal-Wallis test; Figure 4B). Finally, the functionality of CD4$^+$ T cells was

comparable to what was observed for the OPXV-specific T cell responses (Figures S2B and S2E).

CD8$^+$ T cell responses were also able to cross-recognize the MPXV pools. Reactivity peaked at 2 weeks and further declined 5–7 months post-vaccination, with comparable reactivity with the pre-vaccination by AIM (GM ± GSD; pre = 0.05 ± 1.44, 2 weeks = 0.17 ± 4.93, and 5–7 months = 0.06 ± 2.23; p = 0.0014 Kruskal-Wallis test, χ^2 p = 0.0212; Figure 4C) and a less pronounced decline by ICS (GM ± GSD; pre = 0.02 ± 4.42, 2 weeks = 0.03 ± 7.97, and 5–7 months = 0.02 ± 3.96; p = 0.8653 Kruskal-Wallis test, χ^2 p = 0.9961; Figure 4D). The quality of CD8$^+$ T cells was comparable to and driven by IFNγ production, although an increase in IL-2 was also observed (Figures S2D and S2F). Little reactivity was again observed in non-vaccinated subjects for both CD4$^+$ and CD8$^+$ (Figures S1D and S1E).

DISCUSSION

There is an urgent need to understand adaptive immune responses to MPXV in both natural immunity and vaccination. This study is focused on CD4$^+$ and CD8$^+$ T cell responses and peptide MPs to detect these responses. In the context of SARS-CoV-2, we have used three different approaches to design MPs. The first one uses experimentally defined epitopes of highly homologous viral species and typically generates pools with higher activity, but it is limited to described epitopes and may underrepresent less frequent HLAs. A second approach utilizes T cell epitope predictions targeting common HLA alleles.[49] It is limited by the accuracy of bioinformatic predictions, but it can be valuable when little information is available on the actual epitopes recognized in a population and/or when the target pathogen is particularly large so use of complete peptide sets is unfeasible. The third approach utilizes overlapping peptides spanning the entire antigen sequence[37,50] and is the most comprehensive agnostic approach. This approach requires
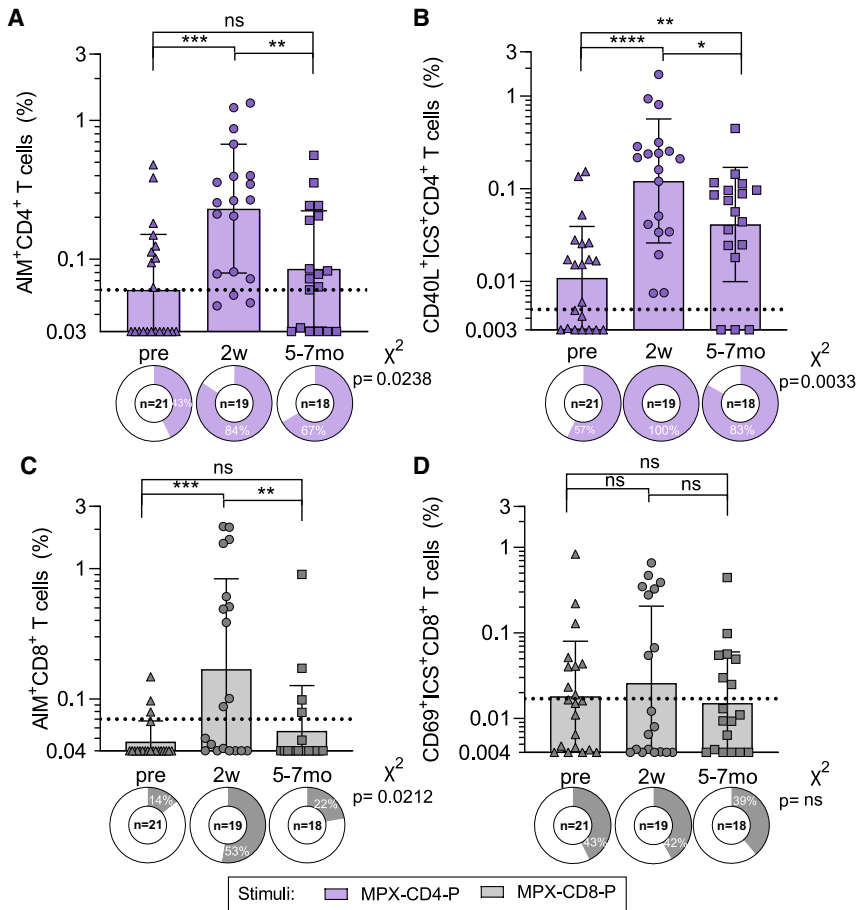
Figure 4. T cell responses able to cross-recognize MPXV MPs after vaccinia vaccination; refers to Figures S1 and S2 and Tables S1–S4

PBMCs from each time point were tested in AIM/ICS assays with predicted MPXV MPs, MPXV-CD4-P (purple), and 5 pools of MPXV-CD8-P1-P5 summed to get the overall MPXV-CD8-P reactivity (gray).

(A and B) MPXV-CD4-P-specific CD4$^+$ T cell reactivity is shown for (A) AIM and (B) ICS.

(C and D) MPXV-CD8-P-specific CD8$^+$ T cell reactivity is shown for (C) AIM and (D) ICS. The y axis of each bar graph starts at the LOD, and the LOS is indicated with a dotted line. Columns represent the geometric mean and error bars indicate the geometric standard deviation. Pie charts below each bar indicate the frequency of positive responders. Mann-Whitney t test was applied to each graph, and p values symbols are shown when significant. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. X^2 test was applied to the frequencies of positivity, and p values are listed on the right of each graph.

and 40 ORFs were required to cover 61% of CD8$^+$ T cell responses. This significant finding suggests that focusing on 10%–25% of the 200+ ORFs typically encoded in pox genomes will still capture the majority of T cell responses. This observation enabled the definition of MPXV-specific MP reagents. The definition of dominant ORFs provided insights into the mechanisms underlying the development of these responses, showing that dominant antigens are predominantly early ORFs, confirming earlier studies.[52] In vaccination, we note that all dominant ORFs were conserved in MVA/JYNNEOS and had orthologs in MPXV. This suggest that the response directed to MPXV induced by MVA should mirror the response induced by previous VACV vaccines, with known clinical efficacy against MPXV in humans.

We developed peptide pools based on experimentally defined Orthopox T cell epitopes or predicted T cell epitopes derived from the most immunodominant ortholog proteins of MPXV, similar to the approach we used for SARS-CoV-2.[37,48] The use of epitope pools has the important potential advantage of obviating the use of infected cells to quantify T cell responses, which is prone to interference by pox-virus expression of immune antagonizing genes[53] and is associated with biosafety concerns.

These Orthopox MPs were validated using PBMC from subjects who received the Dryvax vaccine. Early time points (2 weeks from vaccination) were associated with positive responses in 100% and 63% of the subjects for CD4$^+$ and CD8$^+$ T cells, respectively, and decreased 5–7 months after vaccination. CD4$^+$ and CD8$^+$ T cell responses to the pools of predicted MPXV epitopes were similarly detected in 84% and 52% of vaccinees 2 weeks post-vaccination and also decreased 5–7 months after vaccination. Our results are consistent with the report of Amara et al. that shows a better persistence of CD4$^+$ T cells,

synthesis and testing of many peptides and becomes unfeasible with larger genomes, such as the Orthopoxviridae, which contain ≥200 ORFs. Accordingly, we defined dominant cross-reactive ORFs to generate peptide MPs to measure T cell responses, which we plan to make available to the research community. These reagents were validated by assessing T cell responses induced by Dryvax vaccination in PBMC samples.

Because all current vaccinations against MPXV are based on VACV/MVA, it was important to determine the sequence conservation frequency of previously defined VACV T cell epitopes curated in the IEDB with the current outbreak MA001 strain reference. Overall, the median degree of amino acid sequence conservation was 61%–67%, implying that responses induced by VACV vaccination should recognize ortholog protein sequences in the MPXV genome. McKay and collaborators reported a similar sequence conservation frequency of 70% between MPXV-2022 and the MPXV-CB (Congo Basin) strains.[6] VACV-reactive T cells recognizing MPXV-derived epitopes are not surprising because they cross-react to ectromelia virus epitopes as shown on a mousepox infection model.[51] This observation enabled the definition of Orthopox-specific MP reagents.

The IEDB epitope data were utilized to define dominant ORFs recognized by Orthopox-specific T cell responses. This analysis revealed the remarkable breadth of CD4$^+$ and CD8$^+$ T cell immune responses, consistent with earlier reports.[18,52] Nineteen ORFs were required to cover 67% of CD4$^+$ T cell responses,

with a 2-fold contraction between effector and memory phase, in contrast with the CD8^{+} T cell response that shows a 7-fold contraction.[54] Hammarlund and Slifka reported that the humoral and cellular T cell responses are long-lasting, with a half-life of 8–15 years.[55] This apparent discrepancy can be reconciled considering that in the Hammarlund-Slifka report, the immune response is measured 1+ years after vaccination. Here, we compare pre-vaccination and 6-month post-vaccination samples. Considering cytokine^{+} CD4^{+} T cell responses, which mirror the readout of Hammarlund and Slifka, positive responses observed are 94% 6 months post-vaccination and 71% for pre-vaccinated with a previous history of vaccination in the 5–20 years range. Thus, the % of positivity is comparable. In the context of CD8^{+}, we observed a frequency of response of 30%, whereas Hammarlund, noting several negative donors, reports an overall higher % of responses. Additionally, we observed that human CD4^{+} T cell responses are associated with a large fraction of GZMB-secreting, antigen-specific cells. It was previously reported that the role of CD4^{+} T cells in protection from VACV and MPXV infections outweighs the contribution provided by CD8^{+} T cells in macaques.[29,56] The current data suggest that in addition to their role supporting the development of antibody responses, their longevity and a cytotoxic component could also contribute to a sustained antiviral function of CD4^{+} T cells.

In conclusion, the use of available information related to VACV epitopes in conjunction with bioinformatic predictions points to specific regions that are conserved across several OPXV species, including MPXV, making them suitable for vaccine evaluation. To the best of our knowledge, this is the first demonstration of the use of epitope MPs suitable to characterize vaccine-specific responses and also likely to detect immune responses in the context of MPXV infection and disease.

Limitations and future directions

The cohort selected has the following limitations: cross-sectional sample collection decreases the accuracy of kinetic analysis of responses, a lack of information on childhood vaccination status, and inclusion of healthcare workers previously exposed to occupational vaccination before the Dryvax vaccination administered in relation to this specific study. Antigen selection for MPXV was based on studies performed considering other OPXVs (mainly VACV). Future studies might use overlapping peptide pools spanning various ORFs to define in more detail the ORFs specifically recognized following MPX vaccination or infection. Most previously defined epitopes are focused on more common HLAs, and this might impact detecting responses from individuals with dominant responses restricted by rare HLAs, although this is partially addressed by the prediction of a set of alleles with over 95% coverage in the general population worldwide. The use of peptide pools representing immunodominant viral protein targets may not fully reflect physiological targets. The current analysis did not address antibody responses, which are the dominant correlate of vaccine-induced protection. A strong limitation of the current study is the lack of validation of the MPXV pools in MPXV-infected individuals. This is important because while OPXVs are highly related, their pathogenesis—including processing and presentation of T cell epitopes and being able to evade innate and adaptive immunity—within different

hosts may vary. Future directions will include the following: (1) using the MPs to characterize immune responses in acute and convalescent MPXV natural infection, (2) addressing the Th and memory phenotypes of responding T cells, (3) comparing responses induced by different vaccines, (4) widely disseminating the MPs to the scientific community, and (5) further optimizing these reagents.

STAR★METHODS

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

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AUTHOR CONTRIBUTIONS

Conceptualization, A.G., Y.Z., R.H.S., and A.S.; data curation and bioinformatic analysis, A.G., J.S., and Y.Z.; formal analysis, Y.Z., A.T., and A.G.; funding acquisition, R.H.S. and A.S.; investigation, A.T., M.R.-C., and A.G.; resources, J.M.D. and P.R.; supervision, G.F., A.G., R.H.S., and A.S.; writing, A.G., Y.Z., R.H.S., and A.S.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse anti-human CD8 BUV496 (clone RPA-T8)	BD Biosciences	Cat# 612942; RRID:AB_2870223
Mouse anti-human CD3 BUV805 (clone UCHT1)	BD Biosciences	Cat# 612895; RRID:AB_2870183
Mouse anti-human TNF alpha eFluor450 (clone MAb11)	Life Tech	Cat# 48-7349-42; RRID:AB_2043889
Mouse anti-human CD14 V500 (clone M5E2)	BD Biosciences	Cat# 561391; RRID:AB_10611856
Mouse anti-human CD19 V500 (clone HIB19)	BD Biosciences	Cat# 561121; RRID:AB_10562391
Mouse anti-human CD4 BV605 (clone RPA-T4)	BD Biosciences	Cat# 562658; RRID:AB_2744420
Mouse anti-human IFN gamma FITC (clone 4S.B3)	Invitrogen (Thermo Fisher Scientific)	Cat# 11-7319-82; RRID:AB_465415
Rat anti-human IL-2 BB700 (clone MQ1-17H12)	BD Biosciences	Cat# 566405; RRID:AB_2744488
Mouse anti-human CD69 PE (clone FN50)	BD Biosciences	Cat# 555531; RRID:AB_395916
Mouse anti-human CD134 (OX40) PE-Cy7 (clone Ber-ACT35)	BioLegend	Cat# 350012; RRID:AB_10901161
Mouse anti-human CD137 APC (clone 4B4-1)	BioLegend	Cat# 309810; RRID:AB_830672
Mouse anti-human Granzyme B AF700 (clone GB11)	BD Biosciences	Cat# 560213; RRID:AB_1645453
Mouse anti-human CD154 (CD40 Ligand) APC-ef780 (clone 24-31)	eBioscience (Thermo Fisher Scientific)	Cat# 47-1548-42; RRID:AB_1603203
Biological samples		
Pre-vaccinated donor PBMCs samples	LJI Clinical Core	N/A
Two weeks post vaccinated donor PBMCs samples	LJI Clinical Core	N/A
5-7 months post vaccinated donor PBMCs samples	LJI Clinical Core	N/A
Unexposed donors PBMCs samples	LJI Clinical Core	N/A
Chemicals, peptides, and recombinant proteins		
Brilliant Staining Buffer Plus	BD Biosciences	Cat# 566385; RRID:AB_2869761
Live/Dead Viability Dye eFluor506	Invitrogen (Thermo Fisher Scientific)	Cat# 65-0866-14
Synthetic peptides	TC Peptide Lab	https://www.tcpeptide.com
Deposited data		
OPXV and MPXV peptides	This study	Table S1
OPXV and MPXV megapools (MP)	This study	Table S4
Software and algorithms		
GraphPad Prism 9	GraphPad	https://www.graphpad.com/; RRID:SCR_002798
FlowJo 10.8.1	FlowJo	https://www.flowjo.com/; RRID:SCR_008520
IEDB	Immune Epitope DataBase	https://www.iedb.org; RRID:SCR_006604
ViPR	Virus Pathogen Resource	http://www.viprbrc.org; RRID:SCR_010685

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Alessandro Sette (alex@lji.org).

Materials availability
Aliquots of synthesized sets of peptides identified in this study are available from the lead contact. There are restrictions to the availability of the peptide reagents due to cost and limited quantity.

Data and code availability
All data presented and analyzed in the present study was retrieved from the IEDB (www.IEDB.org) and ViPR (www.viprbrc.org), as described below. The published article includes all data generated or analyzed during this study, and summarized in the accompanying tables, figures and supplemental information. This study uses publicly available algorithms and does not report original code. Any additional information required to reanalyze the data reported in this work paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cohort of VACV vaccinees to assess T cell responses
The characteristics of the study population that donated the banked PBMC utilized for the present study was described previously.[15] Healthy male and female donors between 7 and 62 years of age that had received a vaccinia virus (Dryvax) vaccination as a prophylactic measure, either because of their potential exposure to vaccinia in a laboratory or hospital setting, or because of their enrollment into military and health worker vaccination programs, within one year of providing the blood donation. PBMCs were collected at 2 weeks (n=19) or 5-7 months after vaccination (n=18). In addition, we also utilized two additional control cohorts. For the first control cohort, Pre-vaccination ("pre") PBMC samples (n=21) were collected from a similar cohort of healthcare donors prior to vaccination. Since some of the pre-vaccinated donors had received childhood Smallpox vaccination, a second control cohort of truly unexposed donors (n=15) was enrolled; this cohort consisted of individuals born after 1980, the official year of worldwide eradication of Smallpox and eight years after the United States stopped childhood Smallpox vaccinations. Further, this unexposed cohort had no history of occupational vaccination with Smallpox. Characteristics of the donor cohorts are also summarized in Table S2. Institutional Review Board approval and appropriate consent were obtained for this study.

PBMCs
For all donors in this study, PBMCs were isolated from heparinized blood by density gradient centrifugation with a Histopaque-1077 and cryopreserved in 10% DMSO in FBS prior to long term storage in liquid nitrogen freezers. The PBMC isolation of these specific donors has been described in greater detail by Oseroff et al.[57]

METHOD DETAILS

IEDB analysis of Orthopoxvirus-derived T cell epitopes
Known OPXV-derived T cell epitopes reported in the published literature, or through direct database submission, were identified by searching the IEDB at the end of May, 2022. Queries were performed broadly for the Orthopox genus, using NCBI taxonomy ID 10242, and specifying positive T cell assays. This retrieved 1076 records, from which 31 were removed because responses had not been defined in the context of either MHC class I or class II. For epitopes with responses in the context of class II, the set was further filtered to select epitopes of 12-25 residues, comporting with the canonical size of class II ligands associated with CD4⁺ T cell responses. Epitopes with class I responses were filtered to select those of 8-11 residues, canonical for class I ligands associated with CD8⁺ T cell responses. As a result, a final set of 977 epitopes, including 318 associated with class II responses, and 659 with class I responses, was identified for subsequent analyses. About 70% of the epitope data in this set is derived from the peer-reviewed literature.

Identification of Orthopox T cell epitope homologs in MPXV
The MPXV_USA_2022_MA001 (MA001) isolate (GenBank accession ON563414) was selected as the representative strain of the 2022 MPXV outbreak because MA001 was the first sequence from the 2022 outbreak deposited in GenBank[58] and had been annotated by BV-BRC (www.bv-brc.org). The protein sequences of MA001 were retrieved from the BV-BRC website (=https://www.bv-brc.org/view/Genome/10244.322#view_tab=overview) on May 25, 2022. To identify the MPXV homologs of VACV antigens, a BLAST based homology search was used. All the 26 VACV antigens related to IEDB T cell epitopes except for VP8 and A47 had high identity (>83%) and good length coverage in MA001. With regard to the VP8 antigen we removed 1 nt insertion which caused a frameshift and re-annotated with GATU tool in ViPR (www.viprbrc.org). For the A47 antigen, neither MA001 nor the RefSeq Zaire strain had a good match and it is unlikely to have an MPXV homolog.

To assess the sequence conservation of the OPX-CD8-E and OPX-CD4-E epitope pools in MPXV, a k-mismatch string search program was developed to find all matched sequences of an input epitope. The matched sequences meet the criteria of having the same length as the input epitope, and having at most k mismatched residues in comparison to the input epitope. In case of multiple matches for the same input epitope, the program also picks the optimal match. In order to identify the MPXV protein region homologous to the OPXV T cell epitopes, a k-mismatch string search method was used. Conceptually, the k-mismatch string search program searches through a protein sequence file using a fixed-size sliding window and identifies all windows with a maximum of k-mismatches compared to the input epitope sequence. In identifying OPXV epitope homologs in MPXV, the search pool used included all MA001 protein sequences, while the maximum number of k mismatches was set to be the larger of 20% of the input epitope length and 1, i.e., $k = \max(\text{epitope length} * 0.2, 1)$. We additionally set up a maximum of 2 and 3 mismatches for class I and class II epitopes, respectively.

Besides finding all epitope homologs in proteins, the epitope search program also picks the best match if multiple matches were found. The best match was defined as the one with the smallest number of mismatches, and in case of ties, the one(s) with the least shift in the start coordinate compared with the input epitope.

In validating the epitope search result, two metrics were used: (1) whether the epitope hit resides in a protein homologous to the input epitope's parent protein identified from the pairwise analysis, and (2) whether the start coordinate of the epitope hit was near the start coordinate of the input epitope. In case of a start coordinate shift of 10 or more residues, the sequences of the parent proteins were aligned and then manually examined to see if the match was a false positive. For all epitopes evaluated using these criteria, only one was found to be a false positive following manual curation and was excluded from the downstream analysis.

T cell epitope predictions

Epitope prediction was carried out using the various dominant MPXV ORFs described above (Table S3). For CD4$^+$ T cell epitope prediction, we applied a previously described algorithm that was developed to predict dominant HLA class II epitopes, using a median consensus percentile of prediction cutoff $\leq$ 20 percentile as recommended.[47] For CD8$^+$ T cell epitope prediction, we selected the 12 most frequent HLA class I alleles in the worldwide population,[59,60] using a phenotypic frequency cutoff $\geq$ 6%. The specific alleles included were: HLA-A*01:01, HLA-A*02:01, HLA-A*03:01, HLA-A*11:01, HLA-A*23:01, HLA-A*24:02, HLA-B*07:02, HLA-B*08:01, HLA-B*35:01, HLA-B*40:01, HLA-B*44:02, HLA-B*44:03. HLA class I binding predictions were performed using the IEDB recommended class I prediction algorithm (as recommended in June, 2022) and selecting for each allele the top 1 percentile of peptides based on the total amino acid sequences of the 40 MPXV antigens selected. This initial list of epitopes was then filtered to eliminate redundancies and nested peptides by clustering[61] to a single occurrence, and nested peptides were included within longer sequences, up to 12 residues in length, before assigning the multiple corresponding HLA restrictions for each region.

Peptide synthesis and Megapool preparation

OPXV and MPXV peptides were synthesized as crude material (TC Peptide Lab, San Diego, CA), and then individually resuspended in dimethyl sulfoxide (DMSO) at a concentration of 10–20 mg/mL. Aliquots of all peptides were pooled into megapools (MP) designated as OP-CD4-E, OP-CD8-E, MPX-CD4-P, MPX-CD8-P1, MPX-CD8-P2, MPX-CD8-P3, MPX-CD8-P4, and MPX-CD8-P5. These MPs underwent a sequential lyophilization. The resulting lyocake was resuspended in DMSO to yield a stock solution in which each individual peptide was present at a concentration of 1 mg/mL, as previously described,[37] resulting in a final test concentration of 1ug/mL in the assay after dilution. All peptides and MPs are listed in Tables S1 and S4.

AIM/ICS assay

We performed the combined AIM/ICS assay as previously described.[62] In brief, after thawing, 1–2x10^6 PBMCs per well were cultured with the OPXV- or MXPV- specific peptide MPs (1 μg/mL of each individual peptide contained in the peptide pool). An equimolar amount of DMSO was added to the cells in triplicate wells as a negative control. Phytohemagglutinin (PHA, Roche, 1 μg/mL) was used to stimulate cells as a positive control. Treated cells were incubated at 37°C in 5% CO^2 for 22 hours before the addition of Golgi-Plug containing brefeldin A, Golgi-Stop containing monensin (BD Biosciences, San Diego, CA), and the CD137 APC antibody (2:100, Biolegend, Cat# 309810) for an additional 4-hour incubation. Then the cells underwent membrane surface staining for 30 minutes at 4°C protected from light with Fixable Viability Dye eFluor506 (1:1000, eBiosience, Cat# 65-0866-14) and the following antibodies: CD3 BUV805 (1:50, BD, Cat# 612895), CD8 BUV496 (1:50, BD, Cat# 612942), CD4 BV605 (1:100, BD, Cat# 562658), CD14 V500 (1:50, BD, Cat# 561391), CD19 V500 (1:50, BD, Cat# 561121), CD69 PE (1:10, BD, Cat# 555531), CD137 APC (1:50, Biolegend, Cat# 309810), and OX40 PE-Cy7 (1:50, Biolegend, Cat# 350012). After staining, the cells were fixed with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO), permeabilized with saponin buffer (0.5% saponin [Sigma-Aldrich, St. Louis, MO], 1% bovine serum albumin, and 0.1% sodium azide), and blocked for 15 minutes with 10% human serum (Gemini Bio-Products, Sacramento, CA) in saponin buffer. After blocking, the cells were stained intracellularly for 30 minutes at room temperature with the following antibodies: TNFα ef450(3:100, Life Tech, Cat# 48-7349-42), IFNγ FTIC (1:100, Invitrogen, Cat# 11-7319-82), IL-2 BB700 (1:25, BD, 566405), IFNγ (1:100, Invitrogen, Cat# 11-7319-82), Granzyme B Alexa700 (1:100, BD, 560213), and CD40L APC-eFluor780 (3:100, eBioscience, Cat# 47-1548-42). All samples were acquired on a ZE5 5-laser cell analyzer (Bio-Rad laboratories) and were analyzed with FlowJo software (Tree Star Inc.).

The data was analyzed to establish the Limit of Detection (LOD) and Limit of Sensitivity (LOS) based on all the DMSO-only conditions for AIM and ICS. For ICS these calculations were done on the IFNγ data. The LOD was calculated as twice the upper 95%

confidence interval of the geometric mean and the LOS was calculated as two times the standard deviation from the median. Only responses with Stimulation Index (SI) > 2 were considered significant for AIM (CD4: LOS = 0.06%, SI > 2; CD8: LOS = 0.07%, SI > 2). For ICS, responses with SI>2 were considered significant for CD4$^+$ (LOS = 0.006%) and responses with SI>3 for CD8$^+$ (LOS = 0.017%). For AIM, the CD8$^+$ T cell response to MPXV-CD8-P was calculated by summing the background subtracted, SI>2 and >LOS AIM data. For ICS, the same calculation was performed for the ICS data but considering an SI>3.

QUANTIFICATION AND STATISTICAL ANALYSIS

All the statistical analyses are described separately in each section of the STAR Methods, results and figure legends.

Supplemental information

Defining antigen targets to dissect vaccinia virus

and monkeypox virus-specific

T cell responses in humans

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Supplemental Table 2. Donor cohort vaccinated with Dryvax.

	Unexposed (n=15)	Healthcare workers		
		non-vaccinated (n=21)	2 weeks post vaccination (n=19)	5-7 months post vaccination (n=18)
Age (years)	18-26 [Median =23, IQR = 22]	23-55 [Median =34, IQR = 27]	18-62 [Median =29, IQR = 25]	23-62 [Median =43, IQR = 34]
Gender				
Male (%)	53% (8/15)	48% (10/21)	53% (10/19)	33% (6/18)
Female (%)	47% (7/15)	52% (11/21)	47% (9/19)	67% (12/18)
Days post vaccination	N/A	N/A	13-22 [Median =14, IQR = 14]	141-217 [Median =172, IQR = 142]
Sample collection date	Jun 2019- Dec 2021	Jun 2003-Oct 2005	Jul 2003-Sep 2007	Jun 2003-Aug 2007
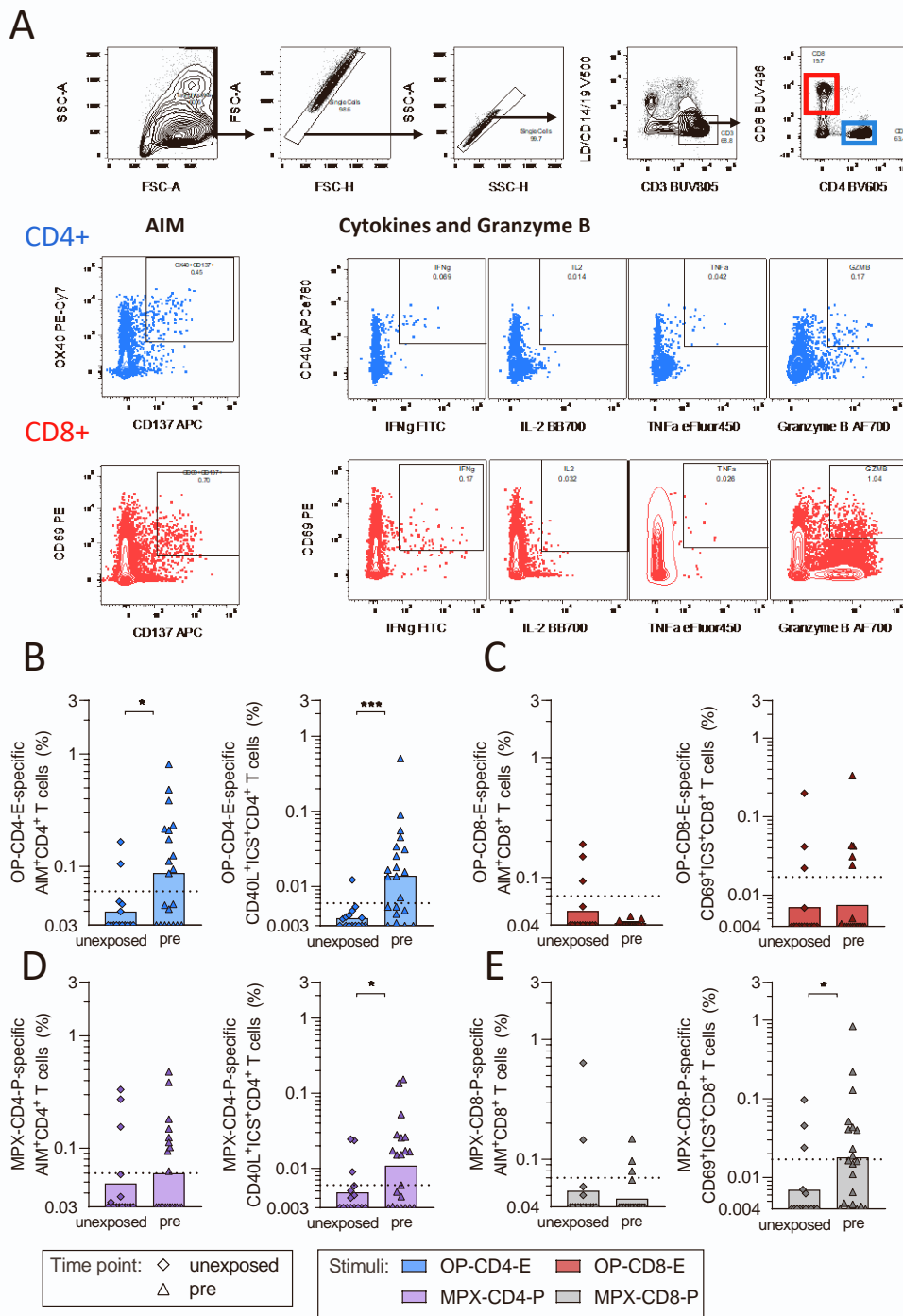
Figure S1. Gating strategy for AIM+ICS assay and T cell reactivity in unexposed and pre-vaccinated individuals.

Related to Figures 1 and 3. (A) Gating strategy for the AIM/ICS assays used in the study. The indicated AIM or intracellular markers were used to assess OPXV or MPXV specific T cell reactivity. For ICS, the total reactivity was calculated from cells positive for IFN γ, IL-2, TNFα or GZMB in combination with CD40L for CD4+ or CD69 for CD8+, with the exception of single positive GZMB cells which were excluded from the CD8 sum. Unexposed individuals (n=15, diamond) and pre-vaccinated healthcare workers (n=21, triangle) were tested in AIM+ICS assays for (B) CD4+ and (C) CD8+ T cell reactivity to the OPXV megapools, OP-CD4-E (blue) and OP-CD8-E (red). The same donors were tested for (D) CD4+ and (E) CD8+ T cell reactivity for MPXV predicted peptide pools, MPX-CD4-P (purple) and MPX-CD8-P (grey) respectively. The Y axis of each bar graph starts at the LOD and the LOS is indicated with a dotted line. Mann-Whitney T test was applied to each graph and p values symbols are shown when significant. * p<0.05, ***p<0.001.

Supplemental Table 3. Summary of immunodominant MPXV proteins based on IEDB epitope orthologs and predicted MPXV T cell epitopes. This summary is based on epitope reassignment to MPXV orthologs and therefore encompasses differences based on different nomenclatures for the various OPXV species listed in Supplemental Table 1.

Cell type	Acc. no.	VACWR antigen	transcription classification[a]	Function[b]	Protein name	Len (OPX)	IEDB epitopes	MPx antigen	MPX protein name	Len (MPX)	# of predicted epitopes	Megapool
CD4	P20642	A10L	Late	structural	Major core protein 4a precursor	891	26	A11L	peg.115| A11L	891	32	MPX-CD4-P
	P21115	B5R	Early	structural	Protein B5	317	19	B6R	peg.159| EEV	317	3	
	P20643	A3L	Intermediate	structural	Major core protein 4b	644	11	A4L	peg.108| A4L	644	25	
	P21010	D5R	Early	regulation	Primase D5	785	11	E5R	peg.96| E5R	785	28	
	QKE61287.1	A26L/A30L	Late	unknown	A-type inclusion protein	725	10	A27L	peg.131| A27L	696	11	
	P20981	L4R	Intermediate	regulation	Core protein VP8	251	9	M4R	peg.0| DNA-binding virion core protein	251	6	
	P33059	H3L	Intermediate	structural	Envelope protein H3	325	9	H3L	peg.87| H3L	324	14	
	P68616	A33R	Early	structural	Protein A33	185	8	A35R	peg.139| A35R	181	3	
	P68445	B7R	Late	structural	Protein B7	182	8	B8R	peg.161	182	5	
	P20504	J6R	Early	regulation	DNA-directed RNA polymerase 147 kDa polypeptide	1286	8	L6R	peg.84| L6R	1286	38	
	P20535	A27L	Intermediate	structural	14 kDa fusion protein	110	7	A29L	peg.133| A29L	110	1	
	P68693	A48R	Early	regulation	Thymidylate kinase	204	7	A49R	peg.152| A49R	204	3	
	I0AZH7/Q8JLB3	D13L	Intermediate	structural	62 kDa protein	551	7	E13L	peg.104| E13L	551	17	
	P21052	F11L	Early	unknown	Protein F11	354	7	C17L	peg.38	354	10	
	I0AZK3/Q8JL90	A24R	Early	regulation	DNA-directed RNA polymerase	1164	5	A25R	peg.129| A25R	1164	29	
	I0AZI2/Q8JLA8	A4L	Early	structural	Core protein	281	5	A5L	peg.109| A5L	281	1	
	I0AZN3/Q8JL68	A56R	Early	structural	Hemagglutinin	319	5	B2R	peg.155| B2R	313	6	
	Q8V523	F8L	Early	unknown	DNA polymerase	1006	5	F8L	peg.52| DNA pol	1006	37	
	P20540	L1R	Late	structural	Protein L1	250	5	M1R	peg.75| M1R	250	7	
CD8	P20979	D1R	Early	regulation	mRNA-capping enzyme catalytic subunit	844	14	E1R	peg.92| E1R	845	71	MPX-CD8-P1
	P20643	A3L	Intermediate	structural	Major core protein 4b	644	13	A4L	peg.108| A4L	644	25	
	P20504	J6R	Early	regulation	DNA-directed RNA polymerase 147 kDa polypeptide	1286	12	L6R	peg.84| L6R	1286	38	
	P21004	B8R	Early	virulence	Soluble interferon gamma receptor B8	272	11	B9R	peg.162| B9R	267	22	
	Q8V523	F8L	Early	unknown	DNA polymerase	1006	11	F8L	peg.52| DNA pol	1006	37	
	P21010	D5R	Early	regulation	Primase D5	785	11	E5R	peg.96| E5R	785	28	MPX-CD8-P2
	P20642	A10L	Late	structural	Major core protein 4a precursor	891	10	A11L	peg.115| A11L	891	32	
	P33059	H3L	Intermediate	structural	Envelope protein H3	325	10	H3L	peg.87| H3L	324	14	
	P20502	I8R	Intermediate	structural	RNA helicase NPH-II	676	10	I8R	peg.64| RNA helicase	676	51	
	I0AZK3/Q8JL90	A24R	Early	regulation	DNA-directed RNA polymerase	1164	9	A25R	peg.129| A25R	1164	29	
	P20640	M1L	Early	unknown	Ankyrin repeat protein M1	472	9	O1L	peg.20| ankyrin-like protein	442	24	
	P68592	A17L	Late	structural	Virion membrane protein A17 precursor	203	8	A18L	peg.122| A18L	204	15	
	I0AZI4/Q8JLA6	A6L	Intermediate	unknown	Virion morphogenesis protein	372	8	A7L	peg.111| A7L	372	22	
	P20998	A23R	Early	regulation	Intermediate transcription factor 3 large subunit	382	7	A24R	peg.128| A24R	382	25	MPX-CD8-P3
	P20535	A27L	Intermediate	structural	14 kDa fusion protein	110	7	A29L	peg.133| A29L	110	1	
	I0AZH7/Q8JLB3	D13L	Intermediate	structural	62 kDa protein	551	7	E13L	peg.104| E13L	551	17	
	P33871	C16L	unknown	unknown	Protein F12	635	7	C18L	peg.39| EEV	635	40	
	P33862	E2L	Early	unknown	Protein E2	737	7	F2L	peg.46	737	55	
	P20501	I7L	Late	structural	Core protease I7	423	7	I7L	peg.63| I7L	423	26	
	P21103	B25R	Late	unknown	Protein C19/B25	259	7	J1R	peg.177| J1R	588	33	MPX-CD8-P4
	P20991	A14L	Late	structural	Virion membrane protein A14	90	6	A15L	peg.119| A15L	90	5	
	I0AZJ7/Q8JL96	A18R	Early	regulation	DNA helicase	493	6	A19R	peg.123| A19R	492	35	
	QKE61287.1	A26L/A30L	Late	unknown	A-type inclusion protein	725	6	A27L	peg.131| A27L	696	11	
	I0AZI2/Q8JLA8	A4L	Early	structural	Core protein	281	6	A5L	peg.109| A5L	281	1	
	P20635	A7L	Late	regulation	Early transcription factor 82 kDa subunit	710	6	A8L	peg.112| A8L	710	50	
	P20986	A8R	Early	regulation	Intermediate transcription factor 3 small subunit	288	6	A9R	peg.113| A9R	292	18	
	P21077	B19R	Early	virulence	Surface antigen S	353	6	B16R	peg.167| B16R	351	25	
	I0AZH6/Q8JLB4	D12L	Early	regulation	Virus termination factor small subunit	287	6	E12L	peg.103| E12L	287	28	
	I0AZD2/Q8JLF2	I4L	Early	regulation	Ribonucleoside-diphosphate reductase	771	6	I4L	peg.60| I4L	771	50	
	P20538	H5R	Early	regulation	Late transcription elongation factor H5	203	6	H5R	peg.89| VLTF-4	210	12	
	P68467	K7R	Early	unknown	Protein K7	149	6	C6R	peg.27	149	7	
	P20989	A12L	Intermediate	structural	25 kDa core protein A12L	192	5	A13L	peg.117| A13L	190	6	MPX-CD8-P5
	P20995	A20R	Early	regulation	DNA polymerase processivity factor component A20	426	5	A22R	peg.126| A22R	426	39	
	P20499	I3L	Early	regulation	Protein I3	269	5	I3L	peg.59| DNA-binding phosphoprotein	269	17	
	P21081	E3L	Early	virulence	RNA-binding protein E3	190	5	F3L	peg.47| dsRNA-binding protein	153	13	
	P21049	E8R	Intermediate	structural	Protein E8	273	5	F7R	peg.51	273	20	
	P21093	O1L	Early	unknown	Protein O1	666	5	Q1L	peg.55	665	67	
	P21026	G5R	Early	unknown	Putative nuclease G5	434	5	G5R	peg.69	434	22	
	P20638	F13L	Intermediate	structural	Envelope phospholipase F13	372	5	C19L	peg.40| palmytilated EEV	372	17	
	P33801	C14L	unknown	unknown	Serine/threonine-protein kinase 2	439	5	C16L	peg.37| Ser/Thr kinase	439	32	

[a] based on Yang et al., 2011 JVI
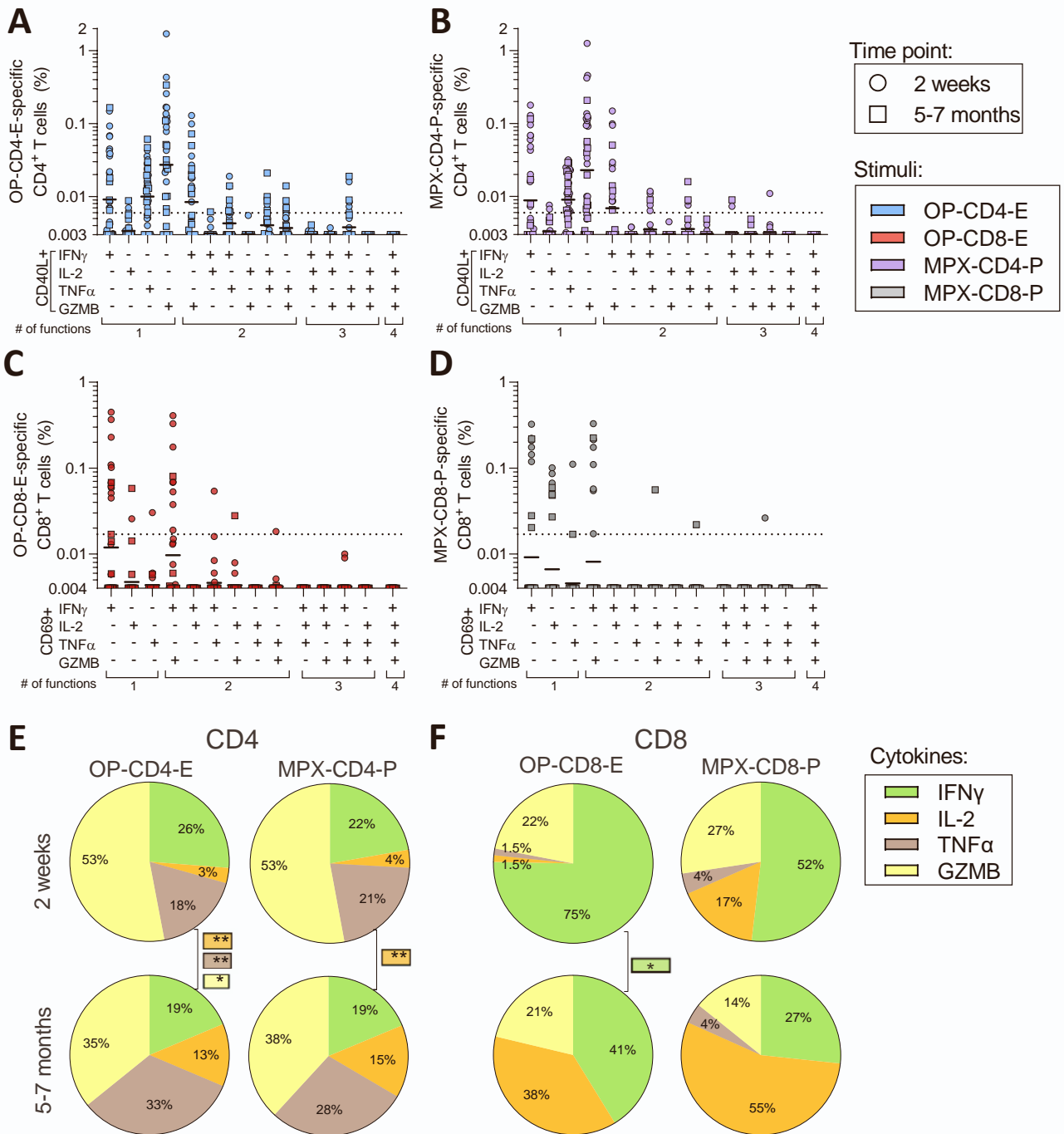
[b] based on Moutftasi et al., 2010 Future Microb

Figure S2. Quality of OPXV and MPXV-specific- T cell responses.
Related to Figures 1 and 3. PBMCs were collected from donors two weeks after vaccination with Dryvax (n=19, circle) and five to seven months after vaccination (n=18, squares) and tested in AIM+ICS assays for T cell reactivity to OPXV and MPXV. (A – B) Boolean gating of (A) OP-CD4-E- or (B) MPX-CD4-P-specific CD4+ ICS reactivity is shown for CD40L+ CD4+ T cells in combination with IFNγ, IL-2, TNFα and GZMB. (C - D) Boolean gating of (C) OP-CD8-E- or (D) MPX-CD8-P-specific CD8 ICS reactivity is shown for CD69+ CD8 T cells in combination with IFNγ, IL-2, TNFα or GZMB. The Y axis of each bar graph starts at the LOD and the LOS is indicated with a dotted line. Contribution of IFNγ, IL-2, TNFα and GZMB to the total of (E) CD4 or (F) CD8+ ICS responses to OP and MPX megapools at two weeks and 5-7 months post vaccination. Mann-Whitney T test was applied to each pie chart and p values symbols are shown when significant. * p<0.05, **p<0.01.