Effect of epitope variant co-delivery on the depth of CD8 T cell responses induced by HIV-1 conserved mosaic vaccines

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To stop the HIV-1 pandemic, vaccines must induce responses capable of controlling vast HIV-1 variants circulating in the population as well as those evolved in each individual following transmission. Numerous strategies have been proposed, of which the most promising include focusing responses on the vulnerable sites of HIV-1 displaying the least entropy among global isolates and using algorithms that maximize vaccine match to circulating HIV-1 variants by vaccine cocktails of optimized complementing sequences. In this study, we investigated CD8 T cell responses induced by a bi-valent mosaic of highly conserved HIVconsvX regions delivered by a combination of simian adenovirus ChAdOx1 and poxvirus MVA. We compared partially and fully mono- and bi-valent prime-boost regimens and their ability to elicit T cells recognizing natural epitope variants using an interferon-γ enzyme-linked immunoospot (ELISPOT) assay. We used 11 well-defined CD8 T cell epitopes in two mouse haplotypes and, for each epitope, assessed recognition of the two vaccine forms together with the other most frequent epitope variants in the HIV-1 database. We conclude that for the magnitude and depth of epitope recognition, CD8 T cell responses benefitted in most comparisons from the combined bi-valent mosaic and envisage the main advantage of the bi-valent vaccine during its deployment to diverse populations.

INTRODUCTION

HIV-1 has a remarkable capacity to adapt to and escape immune responses. Acute infection generates virus variants in numbers more than sufficient to break through the sieve of naturally mounted antibody and T cell responses, leading almost invariably to AIDS when left untreated.1–3 Vaccines are the best solution to HIV-1 control, but to prevent new infections as well as maintain antiretroviral treatment-free virological remission in people living with HIV-1, vaccines must control all of the fittest HIV-1 variants.

Viral proteins encompass both functionally/structurally conserved and less constrained variable regions. For HIV-1, conserved regions contain epitopes that are typically subdominant and therefore underutilized in natural infection due to domination by their more variable non-protective “decoy” counterparts.4–7 To tackle HIV-1 diversity, our working hypothesis postulates that vaccine (re)focusing of killer T cells from the onset of HIV-1 infection/reactivation on the most conserved and therefore vulnerable regions of the virus will slow and control HIV-1.8–11 Such regions are common to most global variants and are harder to mutate. If this vaccine strategy proves effective, the vaccine’s cross-clade reach offers a global deployment.

CD8⁺ killer T cells recognize HIV-1 epitopes of 8–12 aa (most commonly 9) presented by major histocompatibility complex (MHC) class I molecules.12,13 Even the most conserved regions of HIV-1 proteins retain at the epitope level a degree of variability.14 Given that a single amino acid mutation in an epitope can diminish the interaction of a peptide with the MHC molecule or of the loaded MHC-peptide complex with the T cell receptor (TCR) and result in a suboptimal or no stimulatory signal to killer T cells, vaccine immunogens should match as closely as possible the circulating viruses and/or the replication-competent proviruses in the reservoir to maximize the potential for efficient T cell control. A useful way to estimate the suitability of a candidate vaccine is to assess the match between all of the potential 9-mer T cell epitopes (PTEs) across the entire vaccine immunogen and those in the circulating HIV-1 variants/integrated proviruses in the targeted population. For a vaccine design, mosaic immunogens are computed by in silico recombinant of all of the natural HIV-1 strains in the database until the vaccine reaches the highest PTE coverage. The match is further enhanced by using multiple, mutually complementing versions of the same regions in the vaccine cocktail.15 The estimated optimal number of variant mosaics is between two and three, as higher valences start diluting responses to the most frequent epitopes by those recognizing much scarcer epitope variants.16–18 Many potential epitopes in any natural sequence are
RESULTS

The bi-valent conserved mosaic vaccines

The design of the conserved-mosaic immunogens collectively called HIVconsvX was described previously.14,15 Briefly, we used the mosaic algorithm15 to optimize 9-mer PTE coverage over the Gag and Pol proteins for a bi-valent output and selected two and four highly conserved segments in each HIV-1 protein, respectively (Figure 1A). The six selected regions are rich in known highly conserved human CD8 T cell epitopes,37–39 with a total 872 aa, and differ between the two mosics in 84 or 9.6% amino acid residues (Figure S1). Vaccine immunogens HIVconsv1&3&5 and HIVconsv2&4&6/62 were derived from mosaic 1 and mosaic 2, respectively, and the six regions were arranged in six unique orders to minimize induction of T cell responses to unnatural epitopes spanning junctions of adjacent regions that might have been inadvertently generated (Figure 1B). Replication-deficient vaccines ChAdOx1.tHIVconsv1 (C1) plus ChAdOx1.tHIVconsv6 (C6)/ChAdOx1.HIVconsv62 (C62) for priming administration and MVA.tHIVconsv3 (M3) plus MVA.tHIVconsv4 (M4) for boosting (Figure 1C) were used in the present study, whereby C62 (version 2 of HIVconsv6) expresses an immunologically equivalent protein sequence to C6 but uses alternative codons and rearranged region 1 of Gag p24 (Figure 1B) to assure the transgene’s intrinsic stability (unpublished data).

Bi-valent mosaic immunization broadens variant coverage

The aim of this work was to assess experimentally the benefits of the bi-valent mosaic vaccine over a “conventional” single-version immunogen administration. In the BALB/cJ mice, we previously identified 17 H-2\(^{b}\)-restricted peptide pairs, each peptide in a pair corresponding to one mosaic, with net responses greater than 50 spot-forming units (SFU)/10\(^6\) splenocytes in an interferon (IFN)-\(\gamma\) enzyme-linked immunospot (ELISPOT) assay,\(24,40–43\) a qualified and highly sensitive readout for T cell responses. The seven epitopes with the strongest response were narrowed to the optimal length, and their recognition by CD8 T cells was verified using intracellular cytokine staining (ICS) (Table 1). In this study, we first evaluated the immunogenicity of mosaic 1 and mosaic 2 given either singly as C1-M3 or C62-M4 or together in bi-valent regimen C1C62-M3M4. In a pilot experiment (\(n = 3\) mice per group), the frequencies of responsive CD8\(^+\) T cells were determined at three peptide concentrations of 10, 1, and 0.1 \(\mu M\) used for the in vitro restimulation (Figure 2A). The immunodominance of the strongest responses recognizing the VL(V/I)GPTVNI, AMQMLK(D/E)TI, and IFQSSMTKI epitopes as well as the hierarchy of the other subdominant epitopes concurred with previous observations.\(40–42\) The levels of the response were consistently the highest at 10 \(\mu M\) and decreased with dilutions. The 10 \(\mu M\) peptide concentration was used in the subsequent experiment interrogating the benefits of the bi-valent vaccine with more statistical power (\(n = 10\)). The results confirmed the pilot experiment, whereby many frequencies were statistically separable using two-way ANOVA with Dunn’s multiple comparison tests. Each epitope behaved uniquely and, overall, the seven epitopes could be assigned to three distinctive categories (Figures 2B and 2C). In category 1, which included epitopes IFQ(S/C)SMTKI and VYYRDSRP(I/L), one peptide variant was much more stimulatory than the other. Thus, for the IPQ epitope, responses were always the best against the mosaic 1 peptide, while for the VYY epitope, responses to the mosaic 2 variant were the strongest, although mosaic 1 was also stimulatory but less so. In this category, the combined vaccination offered no advantage, but it caused no harm. In category 2 grouping epitopes VL(V/I)GPTVNI and AMQMLK(D/E)TI, mosaic 1 was superior in inducing T cell responses in vivo for recognition of both mosaic-1 and mosaic-2 variants. The bi-valent vaccination enabled enhanced recognition of the weakest variant and yielded comparable induction/recognition of both forms, thus providing enhanced recognition overall. Finally, category 3 encompassed epitopes SPAIFQ(S/C)SM, I(T/I)KIQNFRVYY, and REHLL(K/R)WG. For these epitopes, when only one mosaic was used for vaccination, a vaccine-matched peptide was superior in in vitro stimulation, but when both variants were included in the vaccine, both forms of the peptide were recognized. In this category, bi-valent vaccination provided better variant coverage. Therefore, overall across all seven epitopes, the bi-valent vaccines provided better coverage of variants in five of seven cases, and the bi-valent vaccine did not diminish responses to the best epitope in the other two cases. Comparison of geometric mean T cell frequencies for the 14 tested variant peptides in each animal revealed superiority of the bi-valent regimen over both mosaic 1 and mosaic 2 immunization alone (\(p = 0.0011\) and \(p < 0.0001\), respectively; two-way ANOVA with Dunn’s multiple comparison correction) (Figure 2D).

We also discerned some statistically supported patterns in the data using both a generalized linear model (GLM) and geometric mean permutation test to explore the relationships between level of
response and vaccine across all seven epitopes. There was a strong interaction between the vaccine matched (p < 0.001) and mismatched (p < 0.001) responses, with matched responses being significantly higher (Figures 2A and 2B). Furthermore, mosaic 1 (C1-M3) elicited significantly higher frequencies than mosaic 2 (C6-M4) on matched epitopes (p = 0.009 by GLM and p = 0.03 by permutation test). No significance was reached for the mismatched epitope responses.

**Coverage of non-vaccine epitope variants**

Next, we evaluated the recognition of the additional three most frequent non-vaccine epitope variants listed in the Los Alamos National Laboratory-HIV Sequence Database (LANL-HSD).

For the full view, BALB/cJ mice were immunized using each mosaic alone as C1-M3 (mosaic 1) and C6-M4 (mosaic 2), single mosaic prime-double mosaic boost as C1-M3M4 and C6-M3M4, double mosaic prime-single mosaic boost as C1C6-M3 and C1C6-M4, and the full regimen of C1C6-M3M4. In essence, missing one or two components of the four-vaccine regimen in prime or boost did not dramatically decrease the total number of peptides that individual mice recognized (p = 0.18; ordinary one-way ANOVA) (Figure 3A). The pattern of variant recognition was unique for each epitope. A true "deep" and efficient recognition of variants beyond the mosaic pair was detected for the two strongest epitopes VL(V/I)GPTVNI and AMQMLK(E/D)TI. Epitopes IFQ(C/S)SMTKI, VYYRDSRDP(I/L), and R(E/Q)HLLKWGF recognized at least two other variants, while recognition by SPAIFQ(S/C)SM- and I(T/I)KIQNFRVYY-induced responses were limited, at the tested peptide concentration, to the vaccine forms (Figure S2). As for the contribution of vaccine components, the GLM analysis on the full dataset modeled vaccine induction as a fixed effect and peptide responses as random effect. Despite recognizing marginally more epitopes (Figure 3A), C6-M4 yielded responses of significantly lower frequencies of specific T cells than most other vaccinations (p < 0.008 when comparing to C1-M3M4, C6-M3M4, C1C6-M3, and C1C6-M3M4, and a borderline p = 0.03 for C1C6-M4 < C1C6-M3). All other pairwise comparisons were not statistically significant. When comparing responses to non-matching epitope variants only to avoid matching/mismatching bias using the GLM model,
we found that the C6-M4 was the overall weakest regimen compared to all other combination vaccinations (p < 0.008). When modeling each epitope separately, out of all 16 pairwise comparisons between combination and non-combination vaccinations, 12 times the combination vaccines yielded significantly higher responses. Overall, stronger and deeper responses tended to be favored in combination regimens.

In the BALB/cJ mice, there were two pairs of partially overlapping epitopes, that is, SPA/IFQ and I(T/I)K/VYY. For these epitopes, responses to one peptide always dominated the other overlapping peptide and this was independent of the immunization (Figure 3B). It was noted that in the ELISPOT assay wells containing the same splenocytes and only single restimulating peptide (no peptide competition), this dominance alternated depending on the mosaic origin of the stimulatory peptides. Thus, mosaic 1 peptides were dominated by the IFQ and ITKI responses, while mosaic 2 peptides provided the strongest stimulation for the other SPA- and VYY-specific T cells.

### Comparison of mono-valent and bi-valent priming vaccinations

Due to delay in the C62 vaccine manufacture, the first two clinical trials testing the HIVconsX vaccines will use the C1-M3-M4 regimen (ClinicalTrials.gov: NCT04586673 and NCT04553016). In this study, we evaluated the impact of a mono-valent mosaic prime on the T cell breadth, depth, and magnitude comparing regimens C1-M3-M4, C62-M3-M4, and C1C62-M3-M4 in two strains of mice, BALB/cJ (H-2d) and C57BL/6J (H-2b).

For the initial readout, 10 HIVconsX peptide pools (P1–P10) of overlapping peptide pairs across the entire immunogen were used. In the BALB/cJ mice, all three regimens induced broad responses across most of the pools dominated by pools P1 and P4 (Figure 4A), but the breadths of responses (Figure 4A) and the total frequencies (sums of all 10 pools) (Figure 4B) of the HIVconsX-specific T cells among the vaccinations were not statistically separable. For the seven defined epitopes, immune splenocytes from the same animals were assessed against the vaccine and also non-vaccine epitope variants (Table 2; Figure 4C). GLM found no statistical differences across...
vaccinations in the number of peptides recognized per mouse; however, the magnitude of responses across all epitopes yielded statistically significantly different means \((p = 0.009)\). The permutation test confirmed the GLM finding in that both C1C62-M3M4 and C62-M3M4 regimens resulted in marginal significance, suggesting higher mean responses than C1-M3M4 \((p = 0.02\) and \(p = 0.05\), respectively). The GLM on the full dataset with a vaccine as fixed effect and peptide as random effect yielded the same results: both C1C62-M3M4 and C62-M3M4 induced statistically significantly higher mean responses than did C1-M3M4 \((p = 0.02\) and \(p = 0.03\), respectively), but differences between the two were not statistically separable. The total number of stimulatory HIVconsvX peptides reached a significant difference among the three tested regimens \((p = 0.015\), the Kruskal-Wallis test) \((\text{Figure 4D})\). When looking at each epitope separately \((n = 7)\), no differences were found among vaccinations in the number of recognized variants, although comparing magnitudes of individual responses, three of seven peptides showed a significant difference, and all other comparisons were not significant.

Similarly, in the C57BL/6 mice, broad responses were detected against 10 peptide pools \((P1–P10)\) with a different pattern from the BALB/c mice as per the different haplotype \((\text{Figure 4E})\) and similar overall magnitudes among the three regimens \((\text{Figure 4F})\). By deconvoluting the stimulatory peptide pools, we detected positive responses to 22 peptide pairs, and narrowed the four strongest epitopes with sufficient frequencies of specific T cells to allow for a definition of their optimal length \((\text{data not shown})\); recognition by CD8+ T cells was limited to the index peptide \((\text{Figure 4G})\), with low but detectable re-

**DISCUSSION**

The variability of HIV-1 is daunting, and protective vaccines will have to control effectively every viral variant with fitness above the pathogenic threshold. Currently, we are developing a T cell vaccine strategy that uses two versions \((\text{mosaics})\) of HIV-1 conserved sub-protein regions that are delivered by sequential administrations of the ChAdOx1 and MVA vectors in the ChAdOx1.HIVconsv1 + ChAdOx1.-HIVconsv62-MVA.tHIVconsv3 + MVA.tHIVconsv4 \((\text{C1C62-M3M4})\) regimen. In the present work, we examined this bi-valent mosaic immunization and its impact on the magnitude and depth of induced CD8+ T cell responses by examining the 11 strongest well-defined H-2d and H-2b CD8+ T cell epitopes on the top of the dominance hierarchy. In the BALB/c mice, we could broadly categorize responses into three distinct patterns. In one category, bi-valent immunization was neutral, while in the other two categories, combined bi-valent vaccination provided better recognition of epitope variants. We conclude that ultimately each individual epitope displays a unique pattern of variant recognition.

The HIVconsvX vaccines induced broadly specific CD8+ T cell responses and, for each epitope, the number of recognized variants may increase with overall higher frequencies of epitope-specific T cells. Of the two HIVconsvX mosaics, the most frequent variants were present in mosaic 1 \((\text{Table 2})\). The fact that mosaic 1 \((\text{C1-M3})\) induced overall stronger responses in mice than did mosaic 2 \((\text{C62/C62-M4})\) \((\text{Figure 2B})\) does not necessarily mean that the same mosaic will be the strongest in humans. Given the differences between humans and mice in the presented peptidomes and TCR repertoires, these results are not directly transferable to humans. Also, the evolution of HIV-1 genetic diversity has been driven by the human immune system. Thus, a thorough analysis of the T cell magnitude, breadth, and depth induced by the HIVconsvX vaccines in human volunteers is warranted.

Abdul-Jawad et al.16 established in human leukocyte antigen (HLA)-A2 transgenic mice the superiority of a tri-valent over mono- and bi-valent immunizations using prototype conserved region immunogen HIVconsv. That work concurs with the more subtle differences between the mono- and bi-valent immunizations observed in the present study. One of the aims of the present work was to support the two initial clinical trials with the second-generation HIVconsvX vaccine delivered as C1-M3M4 \((\text{short of the C62 component})\). Mirroring the C1-M3M4 regimen, the differences between the fully and partially bi-valent vaccinations were even more challenging to statistically separate, indicating that the incomplete regimen in humans will be informative about the HIVconsvX vaccine performance.

In the BALB/c mice, we noted a strongly biased competition between partially overlapping epitopes, whereby the excision of one inevitably

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**Figure 2. Comparison of immunizations with either mono- or bi-valent mosaic immunogens**

\((A–D)\) Groups of BALB/c mice \((n = 3, A; n = 10, B–D)\) were vaccinated using the rChAdOx1-MVA regimen with either mosaic 1 alone as C1-M3, mosaic 2 alone as C62-M4, or both mosaics together as C1C62-M3M4 \((C1, \text{ChAdOx1-HIVconsv1}; C62, \text{ChAdOx1-HIVconsv2}; M3, \text{MVA-HIVconsv3}; M4, \text{MVA-HIVconsv4})\). Immune splenocytes were tested against decreasing concentrations of peptide variants of the seven most immunodominant H-2d epitopes in HIVconsvX \((A)\) or were restimulated using 10 µM peptides \((B–D)\) in an IFN-γ ELISPOT assay. \((A\text{ and B})\) Results are shown as background \((\text{no peptide})\)-subtracted median \((\text{range})\) SFU/10^6 splenocytes. Regimens are indicated in the inserted legends. The dotted lines show the arbitrarily chosen limit for a positive response of 50 SFU/10^6 splenocytes. \((C)\) shows the same data as in \((B)\) but plotted according to peptide restimulation to illustrate the three categories of epitope behaviors. Blue and red symbols show responses to mosaic 1 and mosaic 2 epitope variants, respectively. \((D)\) For each mouse, the geometric mean of the frequencies of T cells specific for the 14 tested variant peptides was determined and plotted individually with bars giving the group average. \((B–D)\) Vaccinations with single mosaics were compared to a bi-valent vaccine using two-way ANOVA with Dunnett’s multiple comparison tests. *\(p \leq 0.05\), **\(p \leq 0.01\), ***\(p \leq 0.001\), ****\(p \leq 0.0001\). Color coding of peptides and regimens on the x axis indicates their derivation from mosaic 1 \((\text{blue})\) or mosaic 2 \((\text{red})\). The filled stars in \((A\) and \(B)\) pinpoint the amino acid differences relative to the most frequent peptide variant of mosaic 1.\n
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Intriguingly, mirror results were obtained for pulsing the same immune splenocytes with peptides derived from the non-immunizing mosaic variants (Figure 3B), proving that both overlapping epitopes had primed T cell responses in vivo.

Several processes might collectively influence the outcome of the vector-facilitated immunogen delivery in vivo such as the quality control environment of the endoplasmic reticulum (ER), which may be altered during vaccination by virus infection, competition from vector-derived epitope(s), peptide cross-presentation, engagement of TCRs with MHC irrespective of a peptide, or cross-recognition with previously encountered antigens. Alternatively, subtle ER events of peptide loading might be overridden by higher than physiological peptide concentrations sometimes used for in vitro peptide pulsing.

It is impossible to avoid immunodominance and induce responses recognizing equally all epitopes within a single vaccine immunogen. Due to the interplay of the epitope processing, distinct binding motifs for MHC molecules and differences in the TCR clones recruited into the response, immunodominance is always established, although more “immunodemocratic” strategies were attempted in

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Table 2. Variants of immunodominant H-2d and H-2b HIV-1 epitopes used in this study

| Table 2. Variants of immunodominant H-2d and H-2b HIV-1 epitopes used in this study |
|-----------------------------------|---------------------------------|-----------------|-----------------|
| BALB/cJ                           | C57BL/6J                        |                 |
| Epitope                           | f (%)                           | Epitope         | f (%)           |
| VLVGPTPVNI                        | 77.84                           | REHLLKWGF       | 15.13           |
| VLGPTPVNI                         | 13.06                           | RQHLLRWGF       | 12.99           |
| VLVGPTPINI                        | 4.93                            | RAHLLSWGF       | 11.53           |
| VLVGPTPINI                        | 0.75                            | RQHLLRWGL       | 2.61            |
| VLGPTPINI                         | 0.43                            | RGHLLKWGF       | 2.00            |
| IFQSSMTKI                         | 54.35                           | ITKIQNFRVYY     | 59.39           |
| IFQCSMTKI                         | 20.02                           | IIKIQFRVYY      | 20.13           |
| IFQSSMTRI                         | 6.75                            | ITKIQNFRVYYF    | 2.00            |
| IFQASMTKI                         | 6.64                            | ITNIIKFRVYY     | 1.32            |
| IFQSMIKI                          | 3.96                            | IITIQNFVYY      | 0.89            |
| SPAIFQSSM                         | 63.17                           | VYRDSRDPI       | 52.86           |
| SPAIFQCSM                         | 20.63                           | VYRDSRDPL       | 28.84           |
| SPAIFQASM                         | 6.82                            | VYRDSRDPV       | 5.50            |
| SPSIFQSSM                         | 2.96                            | VYRDNRDPL       | 2.78            |
| SPAIFQYSM                         | 2.14                            | VYFRDSRDPI      | 1.61            |
| AMQMLKDTI                         | 44.09                           | QAISPRTLNA      | 30.14           |
| AMQMLKETI                         | 47.62                           | QALSPTLNA       | 12.90           |
| AMQILKDTI                         | 3.69                            | QSLSPRTLNA      | 7.55            |
| AMQILKETI                         | 1.08                            | QSIISPRTLNA     | 2.25            |
| ALQVLKEV1                         | 0.88                            | QNLSPRTLNA      | 1.85            |
| AMQMLKDAI                         | 0.38                            |                  |                 |
| AMQILKEV1                         | 0.24                            |                  |                 |

Red indicates mosaic 1, blue indicates mosaic 2, and black indicates not represented in the vaccine. Gray boxes highlight the amino acid differences from mosaic 1. f (%), percentage of HIV-1 isolates in the LANL-HSD with the indicated epitope variant (September 2013).
The advantage for the conserved-region vaccines is that the vast majority of dominant and/or newly emerging T cell specificities elicited by the vaccine will be conserved and, therefore, likely target the vulnerable regions of HIV-1 and contribute to protection. This is in contrast to the full-length proteins or natural HIV-1 infection, whereby highly variable immunodominant, but easy-to-escape, decoys may significantly hinder protective effects of the subdominant epitopes.

We use a combination of the ChAdOx1 and MVA vectors to maximize the induction of killer T cells (Figure 1). It is well established that heterogeneous prime-boost regimens of viral vectors delivering a shared immunogen induce more potent immune responses than do repeated immunizations with the same vector carrier. The buildup of anti-vector immunity, mainly antibodies, which dampens the induction of both T cells and antibodies against the passenger transgene product. This is less of a problem for the DNA and mRNA vaccine platforms, which do not express any vector protein(s), and it is of no concern to recombinant protein-based vaccines, which, however, do not stimulate killer T cells very potently. For similar reasons of low pre-existing anti-vector immunity and in contrast to many human adenoviruses, we use the ChAdOx1 vector derived from simian adenovirus Y25, to which antibodies were consistently scarce in tested populations. While repeated homologous immunizations with the ChAdOx1 vector boosted antibody but not T cell responses in recent COVID-19

Figure 3. The depths of variant epitope recognition

Groups of BALB/cJ mice (n = 4) were immunized using various combinations of mono- and bi-valent primes and boosts as shown (C1, ChAdOx1.tHIVconsv1; C6, ChAdOx1.tHIVconsv6; M3, MVA.tHIVconsv3; M4, MVA.tHIVconsv4). The splenocytes from individual mice were restimulated ex vivo with 2 μM variant peptides in an IFN-γ ELISPOT assay. (A) The vaccination regimens are given on the x axis, and the bars show the average total number of vaccine and non-vaccine variant peptides out of 35 that mice responded to with individual mouse data displayed (also see Figure S2). (B) Responses to overlapping epitopes SPA/FQ and I(T/I)K/VYY following immunizations with regimens indicated and color coded in the graph inserts. The results are shown as median (range), and the dotted lines indicate the limit for a positive response of 50 SFU/10⁶ splenocytes. The levels of difference significance are boxed in the graphs. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 (Student’s t test), ns, not significant (p > 0.05). The filled stars depict the amino acid differences relative to the most frequent peptide variant of mosaic 1.
MATERIALS AND METHODS

Animals and Vaccinations

The preparation of vaccines used in this study was described before.14,25 Six-week-old female BALB/c and C57BL/6J mice were immunized intramuscularly under general anesthesia with either a total of 10^8 infectious units (IU) of rChAdOx1(s) or a total of 5 × 10^6 plaque-forming units (PFU) of rMVA(s) 2 weeks apart. On the day of sacrifice, spleens were collected and cells isolated by pressing organs individually through a 70-μm nylon mesh sterile cell strainer (Fisher Scientific) using a 5-mL syringe rubber plunger. Following the removal of red blood cells (RBCs) with RBC lysing buffer Hybri-Max (Sigma-Aldrich, Pool, UK), splenocytes were washed and re-suspended in R10 (RPMI 1640 supplemented with 10% FCS, penicillin/streptomycin, and β-mercaptoethanol) for the ELISPOT assay.

Ethics statement

All procedures and care were approved by the local Clinical Medicine Ethical Review Committee, University of Oxford, and conformed strictly to the United Kingdom Home Office Guidelines under the Animals (Scientific Procedures) Act 1986. Experiments were conducted under Project License 30/3387 held by T.H.

Peptides and peptide pools

All peptides at least 90% pure by mass spectrometry (Ana Spec, San Jose, CA, USA and Synpeptide, Shanghai, China) were dissolved in DMSO (Sigma-Aldrich, Pool, UK) to yield a stock of 10 mg/mL and stored at ~80°C. Four hundred one HIVconsvX-derived 15-mer peptides overlapping by 11 aa (15/11) were divided into 10 pools (P1–P10) of 34–47 individual peptides in a way that variant peptides were always present in the same pool for use in ICS and ELISPOT assays. Pooled and individual peptides were used at a final concentration of 2 μg/mL per each peptide.

The IFN-γ ELISPOT assay

The ELISPOT assay was performed using the mouse IFN-γ ELISPOT kit (Mabtech, Stockholm, Sweden) according to the manufacturer’s instructions. Immune splenocytes were collected and tested separately from individual mice. Peptides were used at 2 μg/mL each unless indicated otherwise, and splenocytes at 2 × 10^5 cells/well were added to 96-well high protein binding Immobilon-P membrane plates (Millipore, UK) that had been pre-coated with 5 μg/mL anti-IFN-γ monoclonal antibody (mAb) AN18 (Mabtech). The plates were incubated at 37°C in 5% CO_2 for 18 h and washed with PBS before the addition of 1 μg/mL biotinylated anti-IFN-γ mAb (Mabtech) at room temperature for 2 h. The plates were then washed with PBS, incubated with 1 μg/mL streptavidin-conjugated alkaline phosphatase (Mabtech) at room temperature for 1 h, washed with PBS, and individual cytokine-producing units were detected as dark spots after a 10-min reaction with 5-bromo-4-chloro-3-idolyl phosphate and nitro blue tetrazolium using an alkaline phosphatase-conjugate substrate (Bio-Rad, Richmond, CA, USA). SFU were counted using an AID ELISPOT reader system (Autoimmun Diagnostika). The frequencies of responding cells were expressed as a number of SFU/10^6 splenocytes.

The ICS assay

Splenocytes were stimulated with peptide at 2 μg/mL or ionomycin and phorbol myristate acetate (PMA) at 2.0 mg/mL and 0.5 mg/mL, respectively, as positive assay controls. Tissue culture media with 1% DMSO was used as a negative control and processed as previously described.24 The following mAb reagents were used: anti-CD3 monoclonal antibody (mAb) AN18 (Mabtech) at room temperature with 5-bromo-4-chloro-3-idolyl phosphate and nitro blue tetrazolium using an alkaline phosphatase-conjugate substrate (Bio-Rad, Richmond, CA, USA). SFU were counted using an AID ELISPOT reader system (Autoimmun Diagnostika). The frequencies of responding cells were expressed as a number of SFU/10^6 splenocytes.

Statistical analysis

Initial statistical analyses were performed using GraphPad Prism version 8.4.3 using non-parametric tests as indicated in the figure legends showing two-tailed p values.
Two statistical approaches were used to enhance data analysis. First, a permutation test, which is non-parametric and makes no assumption of underlying data distribution, was used to test whether vaccine A yields statistically significantly higher responses than vaccine B. This was achieved by first computing the sum of all vaccine A geometric mean responses across all epitopes. Next, the vaccine labels were reshuffled across all mice 1,000 times, and each time the sum of all vaccine A geometric mean responses across all epitopes from the randomized dataset was calculated. A one-sided p value was calculated as the number of times that, out of all randomizations, the sum of all geometric means was greater than the sum of all geometric means from the observed data. Second, a GLM, which is parametric and accounts for variations of vaccine effect across different mice and epitopes, was used to test overall differences in epitope responses across vaccines. Because vaccine effects varied strongly across epitopes and mice, the model was also run within each epitope separately. Note that the variation across mice was small and, when using mice as a random effect, the model diverged. The GLM was also used on each epitope to evaluate within-epitope differences across vaccines and to compare the breadth of responses, where a response was counted as positive when it was over a threshold of 50 SFU/10⁶ sple-nocytes above no-peptide background. The GLM as an overall exploration was run to assess vaccine differences in the per-mouse geometric means of epitope responses across all epitopes (one datum point per mouse, the geometric mean of the responses from that mouse across all epitopes tested), as well as per mouse counts of positive epitope responses (one datum point per mouse, the number of positive epitope responses from that one mouse). For GLM, two-tailed p values were used. For both permutation tests and GLM, p values of less than 0.05 were considered statistically significant.

SUPPLEMENTAL INFORMATION
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AUTHOR CONTRIBUTIONS
Conceptualization, T.H.; methodology, Z.H., N.M., E.G.W., E.E.G., and B.K.; software, all authors; formal analysis, T.H., E.E.G., and B.K.; investigation, N.M. and E.G.W.; resources, T.H.; data curation, N.M., E.G.W., and T.H.; writing – original draft preparation, T.H. and B.K.; writing – review & editing, all authors; supervision, T.H. and B.K.; project administration, Z.H., E.G.W., and T.H.; funding acquisition, T.H. All authors have read and agreed to the published version of the manuscript.

DECLARATION OF INTERESTS
T.H. and B.K. are co-inventors on patents PCT/US14/58422 and EP14846993.5. The remaining authors declare no competing interests.

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