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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 immunospot (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.[1–5] 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.[6–8] 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.[9–11] 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* recombination 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

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rare, because all HIV-1 proteomes include some very rare amino acids at the population level, and if such rare variant epitopes were included in the vaccine, they could potentially attract a large proportion of the T cell response, yet would be unlikely to elicit cross-recognition.[19,20] The mosaic algorithm minimizes the inclusion of rare epitopes by design. Candidate mosaic vaccines were constructed and tested extensively in animal models, where they showed promising induction of $CD4^+$ and $CD8^+$ T cell responses.[14,16–18,20–25] The first phase III efficacy trial testing vaccines with mosaics of full-length Gag, Pol, and Env proteins was launched (ClinicalTrials.gov: NCT03964415). More refined T cell immunogens have also entered clinical evaluation (ClinicalTrials.gov: NCT03844386 and NCT03204617).[10,19]

Our prototype conserved-region T cell vaccine HIVconsv of alternating HIV-1 clade consensus sequences[11] generated encouraging results[26] in clinical trials.[27–35] The upgraded second-generation HIVconsvX vaccines used in this study use algorithm-redefined conserved regions and computed mutually complementing mosaic 1 and mosaic 2 immunogens delivered by a simian adenovirus ChAdOx1 as a bi-valent prime followed by poxvirus MVA as a bi-valent boost.[14] In this study, we interrogate at a high resolution the contribution of each of the two mosaics and each of the four vaccine components to the overall magnitude and depth (number of recognized variants of a single epitope) of vaccine-elicited $CD8^+$ T cell responses in two strains of inbred mice.

RESULTS

The bi-valent conserved mosaic vaccines

The design of the conserved-mosaic immunogens collectively called HIVconsvX was described previously.[14] Briefly, we used the mosaic algorithm[15] 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] total 872 aa, and differ between the two mosaics 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 into 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 genetic stability (unpublished data).

Bi-valent mosaic immunization broadens variant coverage

The aim of this work was to assess experimentally the benefits of the bivalent mosaic vaccine over a "conventional" single-version immunogen administration. In the BALB/cJ mice, we previously identified 17 H-2^d-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)-γ 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 μM used for the *in vitro* restimulation (Figure 2A). The immunodominance of the strongest responses recognizing the VL(V/I)GPTVNI, AMQMLK(E/D)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 μM and decreased with dilutions. The 10 μM 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 Dunnett'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 VYYRDSRDP(I/L), one peptide variant was much more stimulatory than the other. Thus, for the IFQ 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)GPTPVNI 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)WGF. 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 Dunnett'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

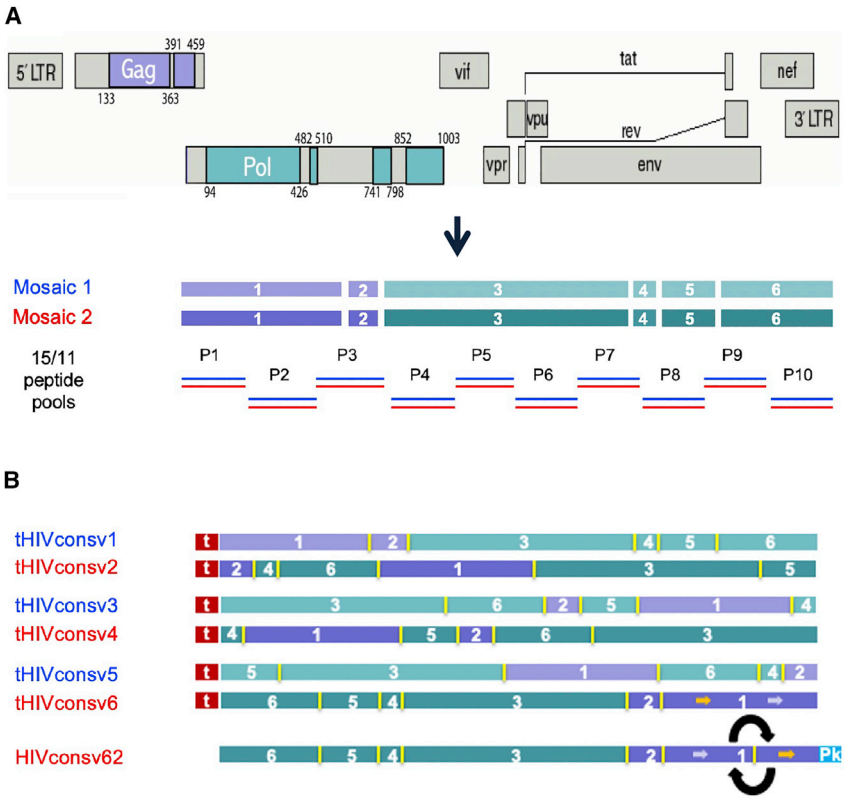


Figure 1. The HIVconsvX vaccine design

(A) Computed bi-valent mosaics were derived from the HIV-1 Gag (regions 1 and 2) and Pol (regions 3–6) proteins using the LANL-HSD HIV-1 group M proteomes as of September 2013.[14] Amino acids of the six conserved regions of mosaic 1 and mosaic 2 achieved between them a perfect match for ~80% of human (HLA-restricted) potential T cell epitopes in these regions. Ten pools of 15-mer peptides overlapping by 11 aa were synthesized across the two HIVconsvX mosaics not crossing the regional junctions. (B) HIVconsvX vaccine immunogens. The six regions of mosaic 1 and mosaic 2 were arranged into six unique orders to avoid immunizing with any junction more than once. HIVconsv62 has the same amino acid sequences as tHIVconsv6, but uses different codons, and rearranged p24. HIVconsv62 and tHIVconsv6 are immunologically equivalent. (C) The caption visualizes the full prime-boost regimen C1C62-M3M4, from which one or two components were subtracted for various reduced regimens. t, human tissue plasminogen activator (tPA) leader sequence; Pk, epitope recognized by monoclonal antibody SV5-Pk (commercially available as V5-tag mAb).[36]

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 (C62-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)[44] (Table 2). 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,

Table 1. Optimal length CD8$^+$ T cell epitopes

Peptide[a]	Peptide pool no.	Mouse strain haplotype	SFU/M[b]	CD3$^+$CD8$^+$ IFN-γ^+ (%)[c]	CD3$^+$CD4$^+$ IFN-γ^+ (%)[d]
AMQMLKDTI (1)	P1	BALB/cJ	3,905	N/D	N/D
AMQMLK*E*TI (2)	P1	H-2^d	3,987	N/D	N/D
VLVGPTPVNI (1)	P4		4,551	7.85	0.28
VL*I*GPTPVNI (2)	P4		4,457	N/D	N/D
IFQSSMTKI (1)	P6		5,136	9.70	0.04
IFQ*C*SMTKI (2)	P6		91	2.71	0.01
SPAIFQSSM (1)	P6		25	N/D	N/D
SPAIFQ*C*SM (2)	P6		2,056	4.26	0.03
REHLLKWGF (1)	P7		573	5.73	0.01
R*Q*HLLRWGF (2)	P7		793	2.50	0.01
ITKIQNFRVYY (1)	P10		1,266	8.48	0.16
I*I*KIQNFRVYY (2)	P10		68	1.25	0.00
VYYRDSRDPI (1)	P10		443	1.04	0.04
VYYRDSRDP*L* (2)	P10		2,751	14.90	0.12
QAISPRTLNA (1)	P1	C57BL/6J	813	3.65	0.09
QA*L*SPRTLNA (2)	P1	H-2^b	29	N/D	N/D
SPVSILDIRQ (1)	P2		2,333	8.26	0.35
SP*T*SILDIRQ (2)	P2		109	N/D	N/D
FLGKIWPSH (1)	P3		149	N/D	N/D
FLGKIWPS*N* (2)	P3		3,229	7.91	0.12
FRELNKKTQ (1)	P5		116	N/D	N/D
FRELNK*R*TQ (2)	P5		2,254	9.03	0.10

Median responses (n = 4) in BALB/cJ and C57BL/6J mice following C1C62-M3M4 vaccination are shown. N/D, not done.
[a]Numbers in parentheses indicate the peptide match to mosaic 1 (1) and mosaic 2 (2), and gray boxes highlight the amino acid differences.
[b]Background-subtracted IFN-γ SFU/10^6 splenocytes.
[c]Background-subtracted frequency of responding CD3$^+$CD8$^+$IFN-γ^+ cells as percentage of all CD3$^+$CD8$^+$ splenocytes.
[d]Background-subtracted frequency of responding CD3$^+$CD4$^+$IFN-γ^+ cells as percentage of all CD3$^+$CD4$^+$ splenocytes.

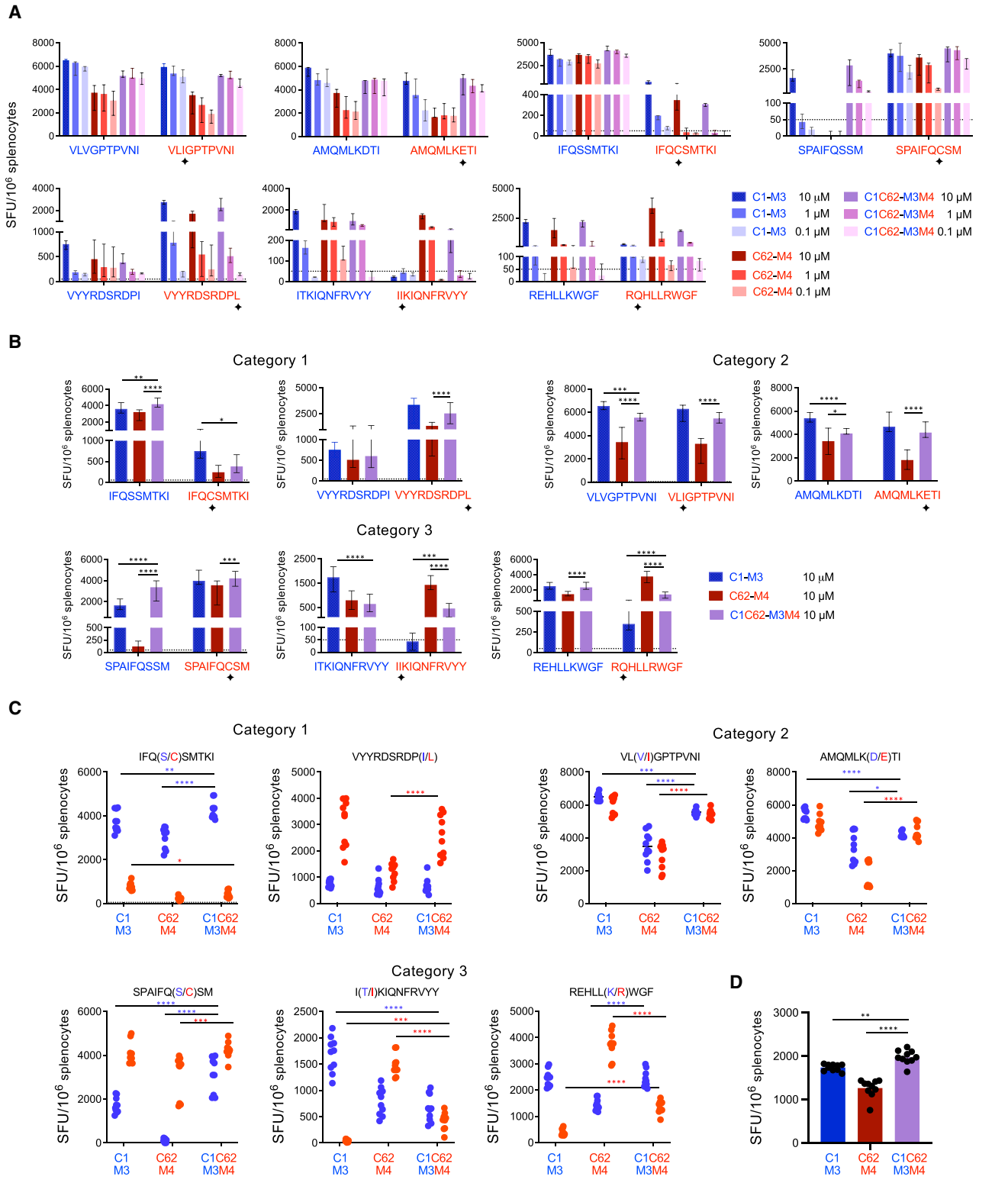
we found that the C6-M4 was the overall weakest regimen compared to all other combination vaccinations ($p < 0.008$). When modeling each epitope separately, out of all 16 pairwise comparisons between combination and non-combination vaccinations, 12 times the combination vaccines yielded significantly 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 HIVconsvX vaccines will use the C1-M3M4 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-M3M4, C62-M3M4, and C1C62-M3M4 in two strains of mice, BALB/cJ (H-2^d) and C57BL/6J (H-2^b).

For the initial readout, 10 HIVconsvX peptide pools (P1–P10) of overlapping peptide pairs across the entire immunogen (Figure 1A) 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 HIVconsvX-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



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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) (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/6J mice, broad responses were detected against 10 peptide pools (P1–P10) with a different pattern from the BALB/cJ mice as per the different haplotype (Figure 4E) and similar overall magnitudes among the three regimens (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 (data not shown); recognition by CD8 T cells was confirmed in ICS (Table 1). Examination of the most frequent variants of the four epitopes (Table 2) revealed a strong recognition limited to the index peptide (Figure 4G), with low but detectable responses to additional variants in the cases of FLGKIWPSN and SPVSILDIRQ. The total numbers of stimulatory variant peptides failed to reach a statistically significant difference among the three regimens (Figure 4H). We concluded that for testing in the initial small phase I trials, the C1-M3M4 regimen is not likely to be severely compromised by missing C62.

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 (mosaics) of HIV-1 conserved sub-protein regions that are delivered by sequential administrations of the ChAdOx1 and MVA vectors in the ChAdOx1.tHIVconsv1 + ChAdOx1.-HIVconsv62-MVA.tHIVconsv3 + MVA.tHIVconsv4 (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-2^d and H-2^b $CD8^+$ T cell epitopes on the top of the dominance hierarchy. In the BALB/cJ 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 (Table 2). The fact that mosaic 1 (C1-M3) induced overall stronger responses in mice than did mosaic 2 (C6/C62-M4) (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 (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/cJ mice, we noted a strongly biased competition between partially overlapping epitopes, whereby the excision of one inevitably

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

(A–D) Groups of BALB/cJ mice ($n = 3$, A; $n = 10$, B–D) were vaccinated using the rChAdOx1-rMVA regimen with either mosaic 1 alone as C1-M3, mosaic 2 alone as C62-M4, or both mosaics together as C1C62-M3M4 (C1, ChAdOx1.tHIVconsv1; C62, ChAdOx1.HIVconsv62; M3, MVA.tHIVconsv3; M4, MVA.tHIVconsv4). Immune splenocytes were tested against decreasing concentrations of peptide variants of the seven most immunodominant H-2^d epitopes in HIVconsvX (A) or were restimulated using 10 μM peptides (B–D) in an IFN-γ ELISPOT assay. (A and B) Results are shown as background (no peptide)-subtracted median (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 (blue) or mosaic 2 (red). The filled stars in (A) and (B) pinpoint the amino acid differences relative to the most frequent peptide variant of mosaic 1.

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

BALB/cJ				C57BL/6J	
Epitope	f (%)	Epitope	f (%)	Epitope	f (%)
VLVGPTPVNI	77.84	REHLLKWGF	15.13	FLGKIWPSH	47.14
VLIGPTPVNI	13.06	RQHLLRWGF	12.99	FLGKIWPSN	14.23
VLVGPTPINI	4.93	RAHLLSWGF	11.53	FLGKIWPSS	8.01
VLVGPTPANI	0.75	RQHLLRWGL	2.61	FLGKIWPSY	2.25
VLIGPTPINI	0.43	RGHLLKWGF	2.00	FLGRIWPSS	1.79
IFQSSMTKI	54.35	ITKIQNFRVYY	59.39	FRELNKKTQ	93.08
IFQCSMTKI	20.02	IIKIQNFRVYY	20.13	FRELNKRTQ	5.10
IFQSSMTRI	6.75	ITKIQNFRVYF	2.00	FRELNRRTQ	0.39
IFQASMTKI	6.64	ITNIQKFRVYY	1.32	FKELNKRTQ	0.14
IFQSSMIKI	3.96	IIQIQNFRVYY	0.89	FGELNKRTQ	0.11
SPAIFQSSM	63.17	VYYRDSRDPI	52.86	SPVSILDIRQ	34.76
SPAIFQCSM	20.63	VYYRDSRDPL	28.84	SPVSILDIKQ	26.25
SPAIFQASM	6.82	VYYRDSRDPV	5.50	SPTSILDIRQ	15.09
SPSIFQSSM	2.96	VYYRDNRDPL	2.78	SPTSILDIKQ	12.58
SPAIFQYSM	2.14	VYFRDSRDPI	1.61	SPVGILDIRQ	1.61
AMQMLKDTI	44.09			QAISPRTLNA	30.14
AMQMLKETI	47.62			QALSPRTLNA	12.90
AMQILKDTI	3.69			QSLSPRTLNA	7.55
AMQILKETI	1.08			QSISPRTLNA	2.25
ALQVLKEVI	0.88			QNLSPRTLNA	1.85
AMQMLKDAI	0.38				
AMQILKEVI	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).

destroys the other.[45] 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.[46–48] 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,[40,48,49] immunodominance is always established, although more "immunodemocratic" strategies were attempted in

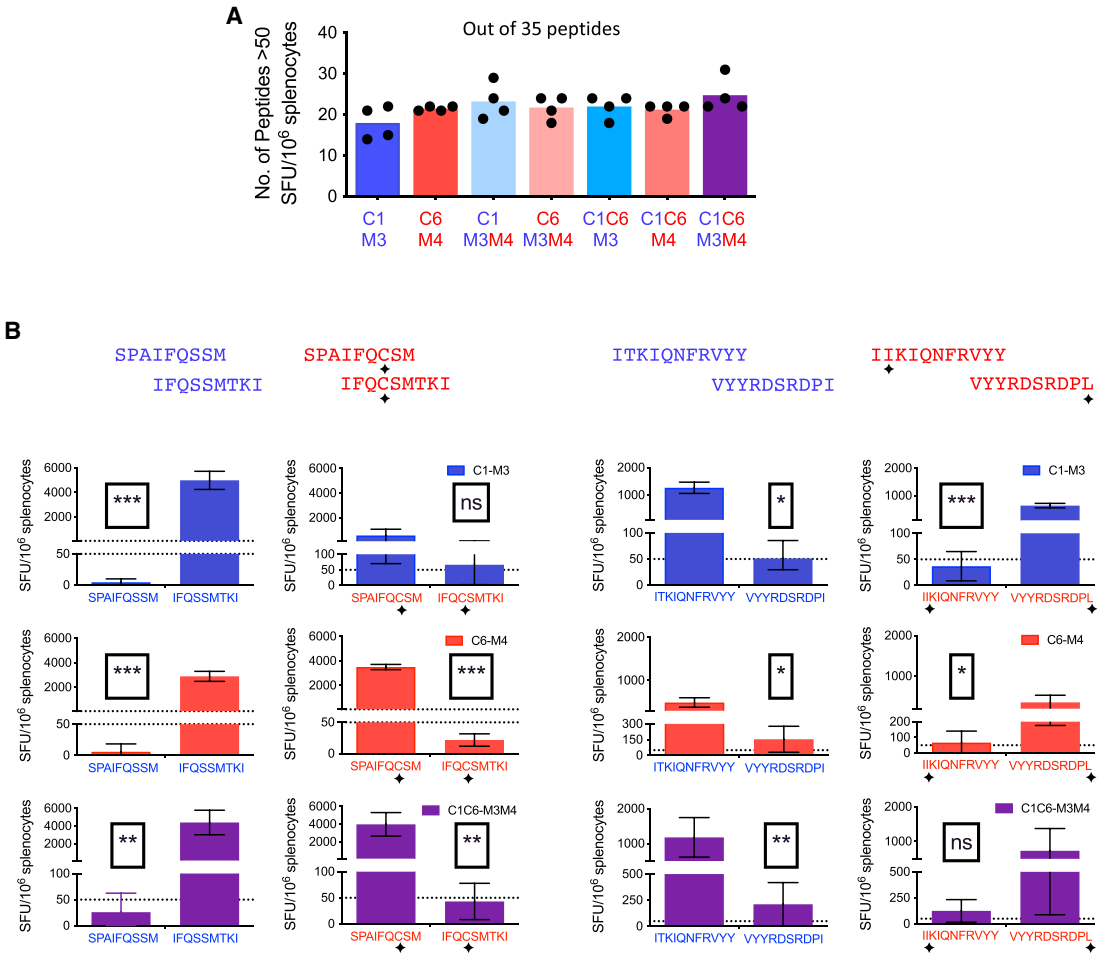
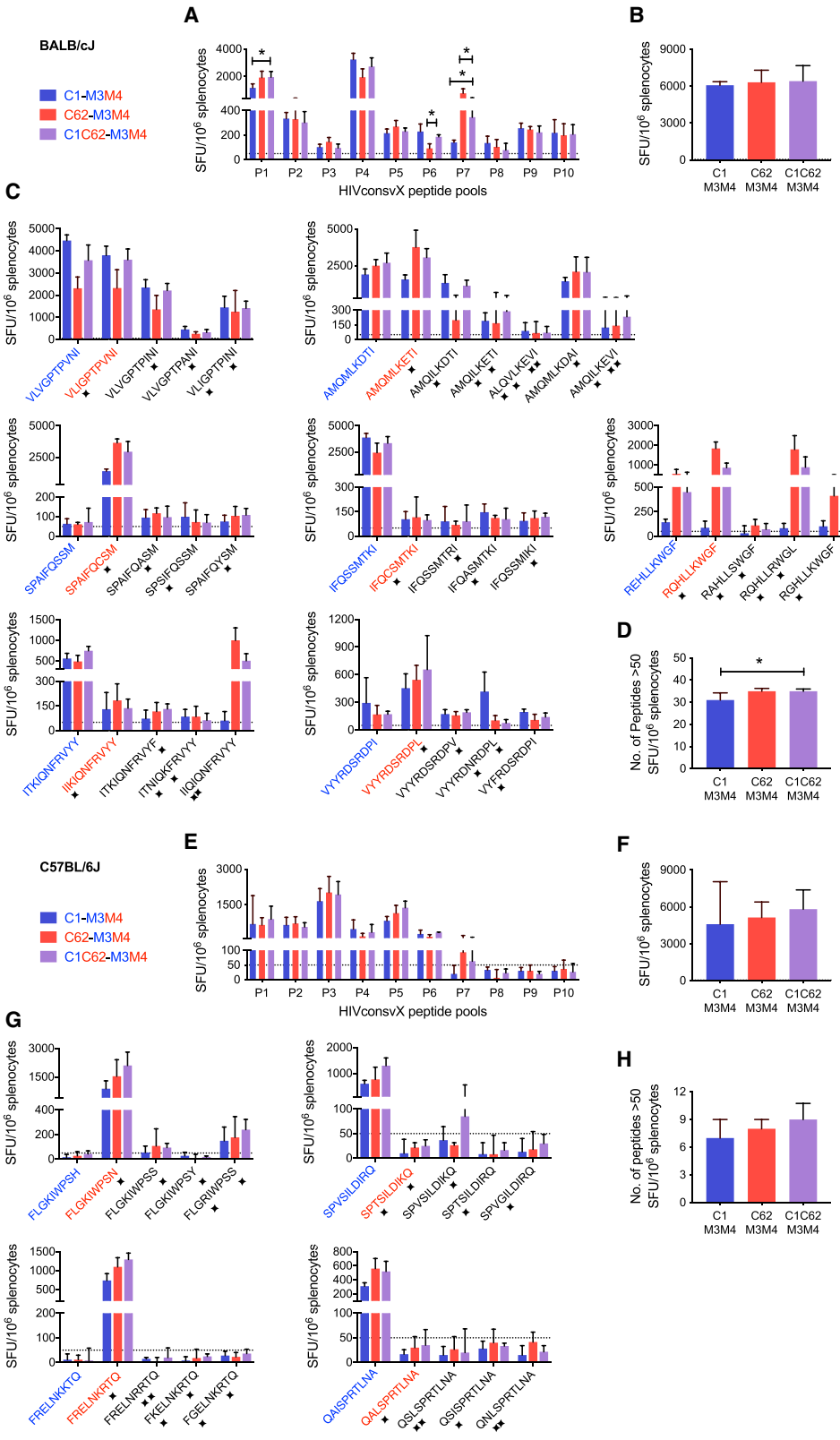


Figure 3. The depths of variant epitope recognition

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/IFQ 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/10^6 splenocytes. The levels of difference significance are boxed in the graphs. *$p \leq 0.05$, **$p \leq 0.01$, **$p \leq 0.001$ (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.

specific cases.[50,51] 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.[28,52–54] This is because of 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.[55–57] For similar reasons of low pre-existing anti-vector immunity and in contrast to many human adenoviruses,[58–61] we use the ChAdOx1 vector derived from simian adenovirus Y25, to which antibodies were consistently scarce in tested populations.[62–64] While repeated homologous immunizations with the ChAdOx1 vector boosted antibody but not T cell responses in recent COVID-19



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trials,[65] extending the time before homologous boost improved the likely antibody-mediated protection against severe disease;[66] whether this was due to the waning anti-ChAdOx1 immunity, maturation of immune responses including T cells, or other factors is under investigation. Thus, the practicality of a single-component vaccine must be balanced against better protection of a heterologous regimen. For HIV-1, simple vaccine solutions remain for the time being elusive.[9,67]

In conclusion, the HIVconsvX vaccines entered human testing in 2019 and a series of clinical trials in both HIV-1-negative individuals and people living with HIV-1 will follow. The present results indicate that missing one vaccine component of the bi-valent prime or bi-valent boost did not dramatically diminish the depth of the elicited $CD8^+$ T cell responses, while fully mono-valent regimens were marginally inferior in several comparisons. We infer that vaccination of diverse human populations with conserved T cell immunogens will benefit from both the bi-valent design and using the full vaccine cocktail.[37,39,68]

MATERIALS AND METHODS

Animals and Vaccinations

The preparation of vaccines used in this study was described before.[14,25] Six-week-old female BALB/cJ and C5BL/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 peridinin chlorophyll protein (PerCP)-eFluor 710, anti-CD8a eFluor 450, and anti-IFN-γ phycoerythrin (PE)-Cy7 (all from eBioscience, San Diego, CA, USA) and anti-CD4 allophycocyanin (APC)/Cy7 (BioLegend, San Diego, CA, USA). Fixed cells were acquired on an LSRII flow cytometer (Becton Dickinson). Examples of dot blots for three epitopes are given in Figure S3.

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.

Figure 4. Comparative recognition of epitope variants induced by mono- and bi-valent primes and bi-valent boost

(A–H) Groups of BALB/cJ (n = 10) (A–D) and C57BL/6J (n = 10) (E–H) mice were immunized using the C1-M3M4 (blue), C62-M3M4 (red), or C1C62-M3M4 (purple) regimens, and the splenocytes from individual mice were restimulated *ex vivo* with 15/11 peptide pools (A, B, E, and F), variant peptides derived from either mosaic 1 (blue), mosaic 2 (red), or variants not present in the vaccine (black) (C, D, G, and H) in an IFN-γ ELISPOT assay. (A) and (E) show responses against 15/11 peptide pools P1–P10; (B) and (F) sum the frequencies of all pools (P1–P10) for individual mice; (C) and (G) show responses to optimal peptide variants; and (D) and (H) give the total numbers of stimulatory optimal peptides for individual mice. (A–C and E–G) Results are shown as no-peptide background-subtracted median (range) SFU/10^6 splenocytes, and the dotted lines indicate the arbitrary limit for a positive response of 50 SFU/10^6 splenocytes. *$p < 0.05$ using the Kruskal-Wallis test with Dunn's multiple comparison post-test. The filled stars highlight the amino acid differences relative to the most frequent mosaic 1 peptide variant.

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 means 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 means 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, 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/10^6 splenocytes 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

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2021.04.018.

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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.

REFERENCES

1. Carrington, M., and O'Brien, S.J. (2003). The influence of HLA genotype on AIDS. Annu. Rev. Med. *54*, 535–551.
2. Dalmasso, C., Carpentier, W., Meyer, L., Rouzioux, C., Goujard, C., Chaix, M.L., Lambotte, O., Avettand-Fenoel, V., Le Clerc, S., de Senneville, L.D., et al.; ANRS Genome Wide Association 01 (2008). Distinct genetic loci control plasma HIV-RNA and cellular HIV-DNA levels in HIV-1 infection: The ANRS Genome Wide Association 01 study. PLoS ONE *3*, e3907.
3. Goonetilleke, N., Liu, M.K., Salazar-Gonzalez, J.F., Ferrari, G., Giorgi, E., Ganusov, V.V., Keele, B.F., Learn, G.H., Turnbull, E.L., Salazar, M.G., et al.; CHAVI Clinical Core B (2009). The first T cell response to transmitted/founder virus contributes to the control of acute viremia in HIV-1 infection. J. Exp. Med. *206*, 1253–1272.
4. Liu, M.K., Hawkins, N., Ritchie, A.J., Ganusov, V.V., Whale, V., Brackenridge, S., Li, H., Pavlicek, J.W., Cai, F., Rose-Abrahams, M., et al.; CHAVI Core B (2013). Vertical T cell immunodominance and epitope entropy determine HIV-1 escape. J. Clin. Invest. *123*, 380–393.
5. Salazar-Gonzalez, J.F., Salazar, M.G., Keele, B.F., Learn, G.H., Giorgi, E.E., Li, H., Decker, J.M., Wang, S., Baalwa, J., Kraus, M.H., et al. (2009). Genetic identity, biological phenotype, and evolutionary pathways of transmitted/founder viruses in acute and early HIV-1 infection. J. Exp. Med. *206*, 1273–1289.
6. Frahm, N., Kiepiela, P., Adams, S., Linde, C.H., Hewitt, H.S., Sango, K., Feeney, M.E., Addo, M.M., Lichterfeld, M., Lahaie, M.P., et al. (2006). Control of human immunodeficiency virus replication by cytotoxic T lymphocytes targeting subdominant epitopes. Nat. Immunol. *7*, 173–178.
7. Frahm, N., Yusim, K., Suscovich, T.J., Adams, S., Sidney, J., Hraber, P., Hewitt, H.S., Linde, C.H., Kavanagh, D.G., Woodberry, T., et al. (2007). Extensive HLA class I allele promiscuity among viral CTL epitopes. Eur. J. Immunol. *37*, 2419–2433.
8. Liu, Y., McNevin, J., Rolland, M., Zhao, H., Deng, W., Maenza, J., Stevens, C.E., Collier, A.C., McElrath, M.J., and Mullins, J.I. (2009). Conserved HIV-1 epitopes continuously elicit subdominant cytotoxic T-lymphocyte responses. J. Infect. Dis. *200*, 1825–1833.
9. Hanke, T. (2014). Conserved immunogens in prime-boost strategies for the next-generation HIV-1 vaccines. Expert Opin. Biol. Ther. *14*, 601–616.
10. Hanke, T. (2019). Aiming for protective T-cell responses: A focus on the first generation conserved-region HIVconsv vaccines in preventive and therapeutic clinical trials. Expert Rev. Vaccines *18*, 1029–1041.
11. Létourneau, S., Im, E.-J., Mashishi, T., Brereton, C., Bridgeman, A., Yang, H., Dorrell, L., Dong, T., Korber, B., McMichael, A.J., and Hanke, T. (2007). Design and pre-clinical evaluation of a universal HIV-1 vaccine. PLoS ONE *2*, e984.
12. Townsend, A.R., Rothbard, J., Gotch, F.M., Bahadur, G., Wraith, D., and McMichael, A.J. (1986). The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. Cell *44*, 959–968.
13. Zinkernagel, R.M., and Doherty, P.C. (1974). Immunological surveillance against altered self components by sensitised T lymphocytes in lymphocytic choriomeningitis. Nature *251*, 547–548.
14. Ondondo, B., Murakoshi, H., Clutton, G., Abdul-Jawad, S., Wee, E.G., Gatanaga, H., Oka, S., McMichael, A.J., Takiguchi, M., Korber, B., and Hanke, T. (2016). Novel conserved-region T-cell mosaic vaccine with high global HIV-1 coverage is recognized by protective responses in untreated infection. Mol. Ther. *24*, 832–842.
15. Fischer, W., Perkins, S., Theiler, J., Bhattacharya, T., Yusim, K., Funkhouser, R., Kuiken, C., Haynes, B., Letvin, N.L., Walker, B.D., et al. (2007). Polyvalent vaccines for optimal coverage of potential T-cell epitopes in global HIV-1 variants. Nat. Med. *13*, 100–106.
16. Abdul-Jawad, S., Ondondo, B., van Hateren, A., Gardner, A., Elliott, T., Korber, B., and Hanke, T. (2016). Increased valency of conserved-mosaic vaccines enhances the breadth and depth of epitope recognition. Mol. Ther. *24*, 375–384.

17. Barouch, D.H., O'Brien, K.L., Simmons, N.L., King, S.L., Abbink, P., Maxfield, L.F., Sun, Y.H., La Porte, A., Riggs, A.M., Lynch, D.M., et al. (2010). Mosaic HIV-1 vaccines expand the breadth and depth of cellular immune responses in rhesus monkeys. Nat. Med. *16*, 319–323.

18. Santra, S., Muldoon, M., Watson, S., Buzby, A., Balachandran, H., Carlson, K.R., Mach, L., Kong, W.P., McKee, K., Yang, Z.Y., et al. (2012). Breadth of cellular and humoral immune responses elicited in rhesus monkeys by multi-valent mosaic and consensus immunogens. Virology *428*, 121–127.

19. Korber, B., and Fischer, W. (2020). T cell-based strategies for HIV-1 vaccines. Hum. Vaccin. Immunother. *16*, 713–722.

20. Santra, S., Liao, H.X., Zhang, R., Muldoon, M., Watson, S., Fischer, W., Theiler, J., Szinger, J., Balachandran, H., Buzby, A., et al. (2010). Mosaic vaccines elicit $CD8^+$ T lymphocyte responses that confer enhanced immune coverage of diverse HIV strains in monkeys. Nat. Med. *16*, 324–328.

21. Barouch, D.H., Stephenson, K.E., Borducchi, E.N., Smith, K., Stanley, K., McNally, A.G., Liu, J., Abbink, P., Maxfield, L.F., Seaman, M.S., et al. (2013). Protective efficacy of a global HIV-1 mosaic vaccine against heterologous SHIV challenges in rhesus monkeys. Cell *155*, 531–539.

22. Hulot, S.L., Korber, B., Giorgi, E.E., Vandergrift, N., Saunders, K.O., Balachandran, H., Mach, L.V., Lifton, M.A., Pantaleo, G., Tartaglia, J., et al. (2015). Comparison of immunogenicity in rhesus macaques of transmitted-founder, HIV-1 group M consensus, and trivalent mosaic envelope vaccines formulated as a DNA prime, NYVAC, and envelope protein boost. J. Virol. *89*, 6462–6480.

23. Kong, W.P., Wu, L., Wallstrom, T.C., Fischer, W., Yang, Z.Y., Ko, S.Y., Letvin, N.L., Haynes, B.F., Hahn, B.H., Korber, B., and Nabel, G.J. (2009). Expanded breadth of the T-cell response to mosaic human immunodeficiency virus type 1 envelope DNA vaccination. J. Virol. *83*, 2201–2215.

24. Moyo, N., Vogel, A.B., Buus, S., Erbar, S., Wee, E.G., Sahin, U., and Hanke, T. (2018). Efficient induction of T cells against conserved HIV-1 regions by mosaic vaccines delivered as self-amplifying mRNA. Mol. Ther. Methods Clin. Dev. *12*, 32–46.

25. Wee, E.G., Ondondo, B., Berglund, P., Archer, J., McMichael, A.J., Baltimore, D., Ter Meulen, J.H., and Hanke, T. (2017). HIV-1 conserved mosaics delivered by regimens with integration-deficient DC-targeting lentiviral vector induce robust T cells. Mol. Ther. *25*, 494–503.

26. Mothe, B., Rosas-Umbert, M., Coll, P., Manzardo, C., Peurtas, M.C., Moron-Lopez, S., et al. (2020). HIVconsv vaccines and romidepsin in early-treated HIV-1-infected individuals: Safety, immunogenicity and effect on the viral reservoir (study BCN 02). Front Immunol. *11*, 418. https://doi.org/10.3389/fimmu.2020.00418.

27. Ahmed, T., Borthwick, N.J., Gilmour, J., Hayes, P., Dorrell, L., and Hanke, T. (2016). Control of HIV-1 replication in vitro by vaccine-induced human $CD8^+$ T cells through conserved subdominant Pol epitopes. Vaccine *34*, 1215–1224.

28. Borthwick, N., Ahmed, T., Ondondo, B., Hayes, P., Rose, A., Ebrahimsa, U., Hayton, E.J., Black, A., Bridgeman, A., Rosario, M., et al. (2014). Vaccine-elicited human T cells recognizing conserved protein regions inhibit HIV-1. Mol. Ther. *22*, 464–475.

29. Borthwick, N., Lin, Z., Akahoshi, T., Llano, A., Silva-Arrieta, S., Ahmed, T., Dorrell, L., Brander, C., Murakoshi, H., Takiguchi, M., and Hanke, T. (2017). Novel, in-natural-infection subdominant HIV-1 $CD8^+$ T-cell epitopes revealed in human recipients of conserved-region T-cell vaccines. PLoS ONE *12*, e0176418.

30. Borthwick, N.J., Lane, T., Moyo, N., Crook, A., Shim, J.M., Baines, I., Wee, E.G., Hawkins, P.N., Gillmore, J.D., Hanke, T., and Pepys, M.B. (2018). Randomized phase I trial HIV-CORE 003: Depletion of serum amyloid P component and immunogenicity of DNA vaccination against HIV-1. PLoS ONE *13*, e0197299.

31. Fidler, S., Stohr, W., Pace, M., Dorrell, L., Lever, A., Pett, S., et al. (2020). Antiretroviral therapy alone versus antiretroviral therapy with a kick and kill approach, on measures of the HIV reservoir in participants with recent HIV infection (the RIVER trial): a phase 2, randomised trial. Lancet *395*, 888–898. https://doi.org/10.1016/S0140-6736(19)32990-3.

32. Hancock, G., Morón-López, S., Kopycinski, J., Puertas, M.C., Giannoulatou, E., Rose, A., Salgado, M., Hayton, E.J., Crook, A., Morgan, C., et al. (2017). Evaluation of the immunogenicity and impact on the latent HIV-1 reservoir of a conserved region vaccine, MVA.HIVconsv, in antiretroviral therapy-treated subjects. J. Int. AIDS Soc. *20*, 21171.

33. Hancock, G., Yang, H., Yorke, E., Wainwright, E., Bourne, V., Frisbee, A., Payne, T.L., Berrong, M., Ferrari, G., Chopera, D., et al. (2015). Identification of effective subdominant anti-HIV-1 $CD8^+$ T cells within entire post-infection and post-vaccination immune responses. PLoS Pathog. *11*, e1004658.

34. Mothe, B., Manzardo, C., Sanchez-Bernabeu, A., Coll, P., Morón-López, S., Puertas, M.C., Rosas-Umbert, M., Cobarsi, P., Escrig, R., Perez-Alvarez, N., et al. (2019). Therapeutic vaccination refocused T-cell responses to conserved regions of HIV-1 in early treated individuals (BCN 01 study). EClinicalMedicine *11*, 65–80.

35. Mutua, G., Farah, B., Langat, R., Indangasi, J., Ogola, S., Onsembe, B., Kopycinski, J.T., Hayes, P., Ashraf, A., Borthwick, N.J., et al. (2016). Broad HIV-1 inhibition in vitro by vaccine-elicited $CD8^+$ T cells in African adults. Mol. Ther. Methods Clin. Dev. *3*, 16061.

36. Hanke, T., Szawlowski, P., and Randall, R.E. (1992). Construction of solid matrix-antibody-antigen complexes containing simian immunodeficiency virus p27 using tag-specific monoclonal antibody and tag-linked antigen. J. Gen. Virol. *73*, 653–660.

37. Mothe, B., Llano, A., Ibarrondo, J., Daniels, M., Miranda, C., Zamarreño, J., Bach, V., Zuniga, R., Pérez-Álvarez, S., Berger, C.T., et al. (2011). Definition of the viral targets of protective HIV-1-specific T cell responses. J. Transl. Med. *9*, 208.

38. Murakoshi, H., Zou, C., Kuse, N., Akahoshi, T., Chikata, T., Gatanaga, H., Oka, S., Hanke, T., and Takiguchi, M. (2018). $CD8^+$ T cells specific for conserved, cross-reactive Gag epitopes with strong ability to suppress HIV-1 replication. Retrovirology *15*, 46.

39. Zou, C., Murakoshi, H., Kuse, N., Akahoshi, T., Chikata, T., Gatanaga, H., Oka, S., Hanke, T., and Takiguchi, M. (2019). Effective suppression of HIV-1 replication by cytotoxic T lymphocytes specific for Pol epitopes in conserved mosaic vaccine immunogens. J. Virol. *93*, e02142-18.

40. Im, E.-J., Hong, J.P., Roshorm, Y., Bridgeman, A., Létourneau, S., Liljeström, P., Potash, M.J., Volsky, D.J., McMichael, A.J., and Hanke, T. (2011). Protective efficacy of serially up-ranked subdominant $CD8^+$ T cell epitopes against virus challenges. PLoS Pathog. *7*, e1002041.

41. Larke, N., Im, E.-J., Wagner, R., Williamson, C., Williamson, A.-L., McMichael, A.J., and Hanke, T. (2007). Combined single-clade candidate HIV-1 vaccines induce T cell responses limited by multiple forms of *in vivo* immune interference. Eur. J. Immunol. *37*, 566–577.

42. Moyo, N., Wee, E.G., Korber, B., Bahl, K., Falcone, S., Himansu, S., Wong, A.L., Dey, A.K., Feinberg, M., and Hanke, T. (2020). Tetravalent Immunogen Assembled from Conserved Regions of HIV-1 and delivered as mRNA demonstrates potent preclinical T-cell immunogenicity and breadth. Vaccines (Basel) *8*, 360.

43. Ondondo, B., Abdul-Jawad, S., Bridgeman, A., and Hanke, T. (2014). Characterization of T-cell responses to conserved regions of the HIV-1 proteome in BALB/c mice. Clin. Vaccine Immunol. *21*, 1565–1572.

44. 2019). Los Alamos National Laboratory HIV Molecular Immunoloy Database (https://www.hiv.lanl.gov).

45. Tussey, L.G., Rowland-Jones, S., Zheng, T.S., Androlewicz, M.J., Cresswell, P., Frelinger, J.A., and McMichael, A.J. (1995). Different MHC class I alleles compete for presentation of overlapping viral epitopes. Immunity *3*, 65–77.

46. Vessey, S.J., Barouch, D.H., McAdam, S.N., Tussey, L.G., Davenport, M.A., O'Callaghan, C.A., Bell, J.I., McMichael, A.J., and Jakobsen, B.K. (1997). Engagement of a T cell receptor by major histocompatibility complex irrespective of peptide. Eur. J. Immunol. *27*, 879–885.

47. Wearsch, P.A., and Cresswell, P. (2008). The quality control of MHC class I peptide loading. Curr. Opin. Cell Biol. *20*, 624–631.

48. Yewdell, J.W. (2010). Designing $CD8^+$ T cell vaccines: It's not rocket science (yet). Curr. Opin. Immunol. *22*, 402–410.

49. Yewdell, J.W., and Bennink, J.R. (1999). Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses. Annu. Rev. Immunol. *17*, 51–88.

50. Mincheff, M. (2009). Immunosurveillance and immunoediting–can the immune response be made more "immunodemocratic"? J. BUON *14* (*Suppl 1*), S89–S96.

51. Rosario, M., Bridgeman, A., Quakkelaar, E.D., Quigley, M.F., Hill, B.J., Knudsen, M.L., Ammendola, V., Ljungberg, K., Borthwick, N., Im, E.J., et al. (2010). Long

peptides induce polyfunctional T cells against conserved regions of HIV-1 with superior breadth to single-gene vaccines in macaques. Eur. J. Immunol. *40*, 1973–1984.

52. Barefoot, B., Thornburg, N.J., Barouch, D.H., Yu, J.S., Sample, C., Johnston, R.E., Liao, H.X., Kepler, T.B., Haynes, B.F., and Ramsburg, E. (2008). Comparison of multiple vaccine vectors in a single heterologous prime-boost trial. Vaccine *26*, 6108–6118.

53. Gilbert, S.C., Schneider, J., Hannan, C.M., Hu, J.T., Plebanski, M., Sinden, R., and Hill, A.V. (2002). Enhanced CD8 T cell immunogenicity and protective efficacy in a mouse malaria model using a recombinant adenoviral vaccine in heterologous prime-boost immunisation regimes. Vaccine *20*, 1039–1045.

54. Lemckert, A.A., Sumida, S.M., Holterman, L., Vogels, R., Truitt, D.M., Lynch, D.M., Nanda, A., Ewald, B.A., Gorgone, D.A., Lifton, M.A., et al. (2005). Immunogenicity of heterologous prime-boost regimens involving recombinant adenovirus serotype 11 (Ad11) and Ad35 vaccine vectors in the presence of anti-ad5 immunity. J. Virol. *79*, 9694–9701.

55. Flynn, N.M., Forthal, D.N., Harro, C.D., Judson, F.N., Mayer, K.H., and Para, M.F.; rgp120 HIV Vaccine Study Group (2005). Placebo-controlled phase 3 trial of a recombinant glycoprotein 120 vaccine to prevent HIV-1 infection. J. Infect. Dis. *191*, 654–665.

56. Pitisuttithum, P., Gilbert, P., Gurwith, M., Heyward, W., Martin, M., van Griensven, F., Hu, D., Tappero, J.W., and Choopanya, K.; Bangkok Vaccine Evaluation Group (2006). Randomized, double-blind, placebo-controlled efficacy trial of a bivalent recombinant glycoprotein 120 HIV-1 vaccine among injection drug users in Bangkok, Thailand. J. Infect. Dis. *194*, 1661–1671.

57. Rerks-Ngarm, S., Pitisuttithum, P., Nitayaphan, S., Kaewkungwal, J., Chiu, J., Paris, R., Premsri, N., Namwat, C., de Souza, M., Adams, E., et al.; MOPH-TAVEG Investigators (2009). Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. N. Engl. J. Med. *361*, 2209–2220.

58. Barnes, E., Folgori, A., Capone, S., Swadling, L., Aston, S., Kurioka, A., Meyer, J., Huddart, R., Smith, K., Townsend, R., et al. (2012). Novel adenovirus-based vaccines induce broad and sustained T cell responses to HCV in man. Sci. Transl. Med. *4*, 115ra1.

59. Barouch, D.H., Kik, S.V., Weverling, G.J., Dilan, R., King, S.L., Maxfield, L.F., Clark, S., Ng'ang'a, D., Brandariz, K.L., Abbink, P., et al. (2011). International seroepidemiology of adenovirus serotypes 5, 26, 35, and 48 in pediatric and adult populations. Vaccine *29*, 5203–5209.

60. Chen, H., Xiang, Z.Q., Li, Y., Kurupati, R.K., Jia, B., Bian, A., Zhou, D.M., Hutnick, N., Yuan, S., Gray, C., et al. (2010). Adenovirus-based vaccines: comparison of vectors from three species of adenoviridae. J. Virol. *84*, 10522–10532.

61. McElrath, M.J., De Rosa, S.C., Moodie, Z., Dubey, S., Kierstead, L., Janes, H., Defawe, O.D., Carter, D.K., Hural, J., Akondy, R., et al.; Step Study Protocol Team (2008). HIV-1 vaccine-induced immunity in the test-of-concept Step Study: a case-cohort analysis. Lancet *372*, 1894–1905.

62. Colloca, S., Barnes, E., Folgori, A., Ammendola, V., Capone, S., Cirillo, A., Siani, L., Naddeo, M., Grazioli, F., Esposito, M.L., et al. (2012). Vaccine vectors derived from a large collection of simian adenoviruses induce potent cellular immunity across multiple species. Sci. Transl. Med. *4*, 115ra2.

63. Dicks, M.D.J., Spencer, A.J., Edwards, N.J., Wadell, G., Bojang, K., Gilbert, S.C., Hill, A.V.S., and Cottingham, M.G. (2012). A novel chimpanzee adenovirus vector with low human seroprevalence: Improved systems for vector derivation and comparative immunogenicity. PLoS ONE *7*, e40385.

64. Dudareva, M., Andrews, L., Gilbert, S.C., Bejon, P., Marsh, K., Mwacharo, J., Kai, O., Nicosia, A., and Hill, A.V. (2009). Prevalence of serum neutralizing antibodies against chimpanzee adenovirus 63 and human adenovirus 5 in Kenyan children, in the context of vaccine vector efficacy. Vaccine *27*, 3501–3504.

65. Ramasamy, M.N., Minassian, A.M., Ewer, K.J., Flaxman, A.L., Folegatti, P.M., Owens, D.R., Voysey, M., Aley, P.K., Angus, B., Babbage, G., et al.; Oxford COVID Vaccine Trial Group (2021). Safety and immunogenicity of ChAdOx1 nCoV-19 vaccine administered in a prime-boost regimen in young and old adults (COV002): a single-blind, randomised, controlled, phase 2/3 trial. Lancet *396*, 1979–1993.

66. Voysey, M., Costa Clemens, S.A., Madhi, S.A., Weckx, L.Y., Folegatti, P.M., Aley, P.K., Angus, B., Baillie, V.L., Barnabas, S.L., Bhorat, Q.E., et al.; Oxford COVID Vaccine Trial Group (2021). Single-dose administration and the influence of the timing of the booster dose on immunogenicity and efficacy of ChAdOx1 nCoV-19 (AZD1222) vaccine: a pooled analysis of four randomised trials. Lancet *397*, 881–891.

67. Kelsoe, G., and Haynes, B.F. (2017). Host controls of HIV broadly neutralizing antibody development. Immunol. Rev. *275*, 79–88.

68. Murakoshi, H., Kuse, N., Akahoshi, T., Zhang, Y., Chikata, T., Borghan, M.A., Gatanaga, H., Oka, S., Sakai, K., and Takiguchi, M. (2018). Broad recognition of circulating HIV-1 by HIV-1-specific cytotoxic T-lymphocytes with strong ability to suppress HIV-1 replication. J. Virol. *93*, e01480-18.