

# Parallel Induction of CH505 B Cell Ontogeny-Guided Neutralizing Antibodies and tHIVconsvX Conserved Mosaic-Specific T Cells against HIV-1

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**The aim of this work was to start collecting information on rational combination of antibody (Ab) and T cell vaccines into single regimens. Two promising candidate HIV-1 vaccine strategies, sequential isolates of CH505 virus Envs developed for initiation of broadly neutralizing antibody lineages and conserved-mosaic tHIVconsvX immunogens aiming to induce effective cross-clade T cell responses, were combined to assess vaccine interactions. These immunogens were delivered in heterologous vector/modality regimens consisting of non-replicating simian (chimpanzee) adenovirus ChAdOx1 (C), non-replicating poxvirus MVA (M), and adjuvanted protein (P). Outbred CD1-SWISS mice were vaccinated intramuscularly using either parallel CM8M (tHIVconsvX)/CPPP (CH505) or sequential CM16M (tHIVconsvX)/CPPP (CH505) protocols, the latter of which delivered T cell CM prior to the CH505 Env. CM8M (tHIVconsvX) and CPPP or CMMP (CH505) vaccinations alone were included as comparators. The vaccine-elicited HIV-1-specific trimer-binding and neutralizing Abs and CD8<sup>+</sup>/CD4<sup>+</sup> T cell responses induced by the combined and comparator regimens were not statistically separable among regimens. The Ab-lineage immunogen strategy was particularly suited for combined regimens for its likely less potent induction of Env-specific T cell responses relative to homologous epitope-based vaccine strategies. These results inform design of the first rationally combined Ab and T cell vaccine regimens in human volunteers.**

## INTRODUCTION

The best control of HIV-1 infection achieved by active immunization will likely require a combined induction of broadly neutralizing antibody (bnAb) and potent anti-HIV-1 T cell responses.<sup>1</sup> Isolation and characterization of hundreds of bnAbs from naturally infected subjects illustrated that the human immune system is capable of generating antibodies of the correct bnAb specificities.<sup>2–6</sup> Nevertheless, elicitation of such antibodies has been challenging because of immune tolerance,<sup>1</sup> conformational masking, glycan shielding, and the unusually long maturation process involving many somatic mutations over

2–5 years to reach bnAb activity.<sup>3,4</sup> Through iterative improvements, the bnAb vaccine field is progressing toward a design of stabilized trimeric Env immunogens rigid enough to allow efficient affinity maturation.<sup>7</sup> At the same time, vaccine designs based on either epitope or guided evolution of Ab specificity along a B cell lineage toward a broad neutralization of tier-2 (difficult-to-neutralize yet majority of the transmitted species) viruses, which promote affinity maturation of otherwise disfavored B cell lineages,<sup>8,9</sup> are gradually getting more successful, at least in animal models.<sup>10</sup>

As for T cells, the natural T cell response to the entire HIV-1 proteome in an infected individual does not protect despite the wealth of data pointing to the CD8<sup>+</sup> T cells' ability to impose a selective pressure on HIV-1<sup>11–13</sup> as well as control experimental simian/simian-HIV (SIV/SHIV) infections<sup>14,15</sup> and association of some CD4<sup>+</sup> T cell responses with HIV-1 disease control.<sup>16–18</sup> It follows that the total T cell response cannot be easily correlated to a good clinical outcome, because not all naturally induced T cells are protective. After many years of research, the T cell field is coming to a realization that vaccine-elicited responses will need to be targeted toward particular vulnerable and, therefore, protective determinants on the virus, while avoiding as many as possible of the distractive non-protective “decoys.”<sup>19–21</sup> Thus, immunogens have been designed ranging from the most conserved full-length proteins<sup>22</sup> to conserved regions,<sup>13,23</sup> conserved epitopes (elements),<sup>24–26</sup> and even conserved (networked) amino acids,<sup>27</sup> the last two being delivered as a string of epitopes. In essence, development of bnAb and effective T cell vaccines requires distinct, highly specialized areas of skills carried out in separate expert laboratories, and the end products will need to be combined into one vaccination protocol at least semi-rationally to avoid compromising each other's efficiency.<sup>28</sup>

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In contrast to the highly variable, flexible, and easy-to-fall-apart native Env trimers on the surface of HIV-1 virions and the naturally shed gp120, for induction of antibodies, the more rigid the immunogen, the more efficiently it drives affinity maturation.<sup>29–31</sup> The prototype of a native-like Env-like immunogen is the BG505 SOSIP.6R.664 trimer, which is subject to relentless iterative engineering with the aim to maximize stabilization of its structure. Furthermore, lessons learned from the antibody-HIV-1 co-evolution within infected individuals following transmission<sup>32–35</sup> suggested that it might be beneficial to engage the unmutated common ancestor (UCA) of the target antibody B cell lineage in the human B cell receptor repertoire and/or employ ontogeny-based HIV-1 Env subunits to guide affinity maturation toward the bnAb specificity. The native-like stabilized SOSIP (stabilizing mutations in the trimer immunogen using additional disulfide bond [SOS] and isoleucine-to-proline [I559P] substitution) trimers and guided immunization married into the EnvSeq-1 series of isolated CH505 Env immunogens, which induced plasma antibodies capable of neutralizing heterologous tier-2 HIV-1 strains in a small subset of experimental animals, proving the feasibility of the concept.<sup>30</sup> This strategy as sequential gp120s has now entered a clinical evaluation (NCT03220724).

As for the T cell vaccines, we have pioneered a strategy, which (re) focuses HIV-1-specific T cells on the most functionally conserved regions of the virus, which contain both CD8<sup>+</sup> and CD4<sup>+</sup> T cell targets.<sup>20,23</sup> During natural infection, conserved regions are subdominant to the hypervariable, easy-to-change and therefore less protective “decoy” epitopes.<sup>36–40</sup> Delivered by simian adenovirus and poxvirus modified vaccinia virus Ankara (MVA), the first-generation conserved clade consensus vaccines were tested in eight trials<sup>36,41–47</sup> and showed high immunogenicity,<sup>36,41,44,46–48</sup> replication control *in vitro* of four major HIV clades, A, B, C, and D,<sup>47</sup> and in a pilot study in combination with latency-reverting agent romidepsin produced a signal of viremic control during monitored antiretroviral treatment (ART) pause in 36% of early treated vaccine recipients (B. Mothe et al., 2017, Conference on Retroviruses and Opportunistic Infections, abstract). The vaccines with vorinostat were also used in the first randomized blind “kick-and-kill” trial RIVER.<sup>42</sup> Currently, we are developing the second-generation immunogens improved by bioinformatics-assisted redefinition of conserved regions, inclusion of protective and conserved epitopes, and maximizing match to global HIV-1 variants by a bivalent mosaic design.<sup>13</sup> The protective potential of the six conserved HIV-1 regions selected for the second-generation immunogens was demonstrated by studies in around 200 treatment-naive naturally HIV-1-infected (not vaccinated) Japanese patients.<sup>13,49,50</sup> Because HIV-1 control is likely determined early rather than during the chronic phase of infection and progressors do not spontaneously change into controllers during the chronic stage, being able to target HIV-1 conserved regions from the very first HIV-1 exposure and/or reactivation may be critical for virus control. Targeting vulnerable determinants on HIV-1 from the very first virus exposure is the paradigm of our strategy.<sup>20</sup>

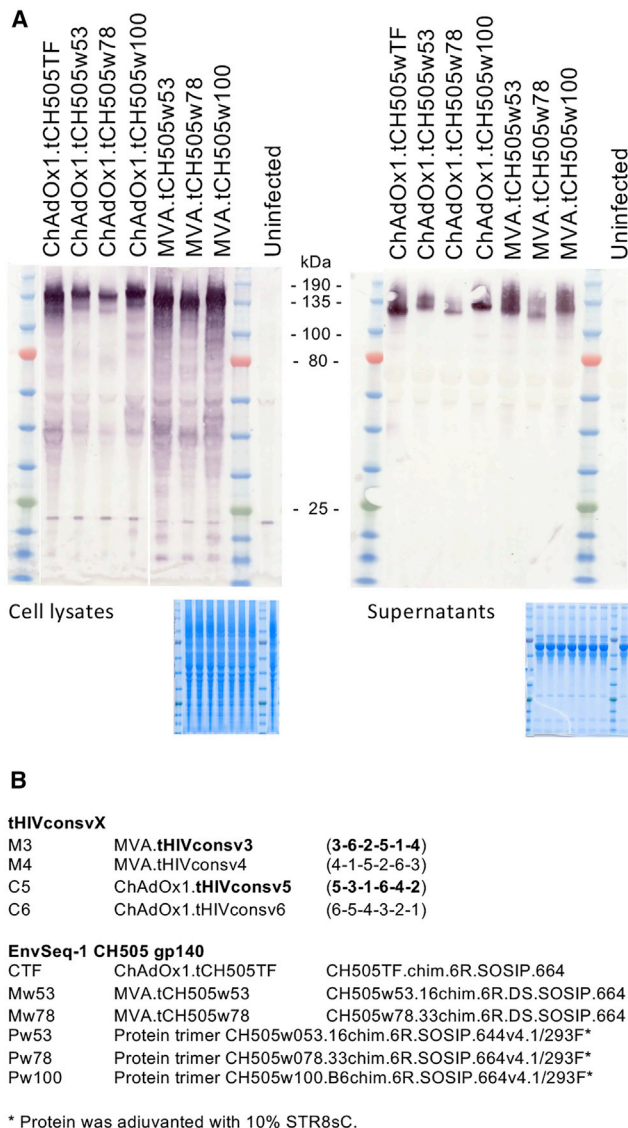
In the past, we searched for an optimal regimen combining the first-generation conserved-region T cell vaccines with an early uncleaved version of the BG505 Env<sup>28</sup> and for any possible benefits of delivering the BG505 SOSIP.6R.664 trimer using viral vectors based on simian adenovirus and MVA.<sup>51</sup> Here, we combined for the first time the two highly promising strategies of antibody-lineage immunization and conserved-mosaic T cell design and induced in outbred CD1-SWISS mice parallel HIV-1-neutralizing antibodies and T cell effectors. Ramifications of these early results for rational, optimized T and B cell vaccine delivery are discussed.

## RESULTS

### Construction of Viral Vectors Expressing the EnvSeq-1 Sequential Isolates of CH505 SOSIPs

In the course of this work, two non-replicating engineered viral vaccine vectors, ChAdOx1 and MVA, were employed. ChAdOx1 was derived from group E simian adenovirus Y25 of a chimpanzee origin and was chosen for low human seroprevalence.<sup>52</sup> MVA originated directly from Professor Mayr, passage 575 dated 14-12-1983. The four individual EnvSeq-1 CH505 HIV-1 vaccine Envs as gp140 used in this study were designated CH505TF (transmitted-founder virus), CH505w53, CH505w78, and CH505w100, according to the number of weeks from transmission.<sup>30</sup> The native HIV-1 Env leader sequences were replaced by that of the human tissue plasminogen activator commonly used to increase transgene product expression,<sup>53–57</sup> and the SOSIP.6R.664 trimers were chimeric for the BG505 gp41-derived stalks<sup>58,59</sup> (Figure S1). The ORFs coding for the four modified Envs were synthesized and inserted into both vector genomes with the exception of the TF virus Env, which was not used for MVA, because MVA is a known weak primer of immune responses.<sup>51,60</sup> The vaccines did not contain a gene expressing the Furin protease, and thus the Env trimer maturation by cleavage relied on endogenous Furin-family proteases of the infected cells.<sup>61</sup> All newly constructed vaccines passed the internal QC requirements based on the DNA sequence of the transgene, flank-to-flank PCR across the inserted DNA fragment, and, for adenoviruses, integrity of the genomic DNA by diagnostic digest pattern analysis using three restriction endonucleases. For the recombinant MVA virus stocks, the absence of parental virus MVA.RFP was confirmed by PCR. Env expression was readily detected using western blots of both lysates and supernatants of virus-infected HeLa cells (Figure 1A).

The CH505 Env trimers were prepared by transient transfection of HEK293T cells.<sup>30</sup> Thus, using biolayer interferometry and consistent with native-like envelope structures, the trimeric envelopes bound to neutralizing but lacked binding to non-neutralizing antibodies. Trimeric envelopes were also verified by size of the protein using analytical size exclusion chromatography and blue native polyacrylamide gel electrophoresis. The negative stain electron microscopy was employed to visualize over 10,000 trimers per preparation, and the mean percentages of native-like trimers across different lots of the CH505w53.16, CH505w78.33, and CH505w100.B6 proteins were 98.8%, 100%, and 100%, respectively.



**Figure 1. Vaccines Used in This Study**

(A) Genes coding for sequentially isolated CH505 Envs of EnvSeq-1 were synthesized and cloned into viral vaccine vectors ChAdOx1 and MVA. The CH505 isolates were from the transmitted-founder virus (TF) and viruses isolated on weeks 53, 78, and 100. The natural leader sequences were replaced with a 22-amino-acid-long derivative of the leader sequence of the human tissue plasminogen activator designated as "t." The Env open reading frame was further modified to form a SOSIP trimer, carried 6Rs for an efficient cleavage by Furin proteases and was terminated after amino acid 664 to secrete covalently linked gp140 with the gp41-derived portion replaced with that of BG505. The vaccine stocks were prepared, and the expression of the transgene products was confirmed in the cell lysates (left) and supernatants (right) of vaccine-infected HeLa cells using a western blot analysis. The correct size bands of monomeric gp140 Envs were detected using mAb 3B3<sup>70</sup> and anti-mouse secondary mAb conjugated to HRP followed by chemiluminescence. The smaller gel images stained with Coomassie blue below the western blots show the protein load prior to transfer onto the nitrocellulose membrane. (B) The list of T cell tHIVconsvX and B cell CH505 EnvSeq-1 vaccines used in this study. The tHIVconsvX immunogens consist of six conserved regions

Construction of the T cell tHIVconsvX vaccines and their immunopotencies were described elsewhere.<sup>13,62,63</sup> For clarity, all vaccines used in this study are listed in Figure 1B.

### Rationale for the Immunization Regimens

The main objective of this study was to optimize co-delivery of the Ab and T cell vaccines for immunopotency. For antibody induction, we immunized once with each of the four sequential Env gp140 SOSIP trimer isolates from weeks 0 (TF), 53, 78, and 100 and used a 4-week interval between immunizations. Based on our rabbit results with BG505 Env SOSIP, whereby recombinant ChAdOx1 (C) was a more efficient prime than adjuvanted protein (P),<sup>51</sup> in groups 1, 4, and 5, we primed with C and followed by three protein boosts in CPPP; this regimen induced weak BG505 Env-specific T cells.<sup>28</sup> We also wondered whether or not a recombinant MVA (M) boost between C and P in CMMP in group 2 would be more potent for induction of binding and/or neutralizing antibodies.<sup>51</sup> Group 3 served as a comparator for induction of T cells alone using a well-documented potent regimen CM8M (B. Mothe et al., 2017, Conference on Retroviruses and Opportunistic Infections, abstract),<sup>36,41,42,46-48,64,65</sup> whereby the number indicates the gap in weeks between the two M administrations. Our main concern for combining Ab and T cell vaccines was that we would induce strong Env-specific T cells, which would be counterproductive to our refocusing of T cells away from highly variable non-protective Env epitopes toward conserved Gag and Pol regions. Thus, group 4 intended to first expand tHIVconsvX T cells with CM, immunize with Envs and, for the finish, co-administer a final T cell focusing M along with the last Env protein boost in CM16M; this was the longest immunization protocol, taking 20 weeks. In contrast, the last group 5 tested a straight parallel immunization with CM8M for T cells and CPPP for antibodies. Groups of eight mice of the outbred CD1-SWISS stock were used to make the experiments more realistic than inbred strains. All regimens are depicted in Table 1.

### Administration of tHIVconsvX Vaccines Does Not Compromise the CH505 EnvSeq-1 CPPP Potency

For induction of CH505 Env-specific antibody, we first assessed the serum antibody binding titer to Pw100, and several observations were made. Thus, ChAdOx1.CH505FT administration primed weakly CH505 Env-specific antibodies in all groups except for group 4, where the ChAdOx1.CH505FT priming was compromised presumably by the earlier exposure to the ChAdOx1.tHIVconsv5&6 vaccines (Figure 2A). Because P with the TF Env was not used as the first vaccination in any of the Ch505 EnvSeq1 regimens, we could not compare the PTF prime with the ChAdOx1.CH505FT prime in this model. The overall kinetics of the group median endpoint titers was not separable (Figure 2B). For peak median titers, the EnvSeq-1

(two of Gag and four of Pol) presented as mosaic 1 (tHIVconsv3 and 5) (bold) and mosaic 2 (tHIVconsv4 and 6), each using a unique region order indicated in the brackets. For the CH505w53.16, CH505w78.33, and CH505w100.B6 vaccine preparations, the mean percentages of native-like trimer across different lots of the proteins were 98.8%, 100%, and 100%, respectively.

**Table 1. Immunization Schedules**

Grp	n = 8	Regimen	w0	w2	w4	w8	w10	w12	w14	w16	w18	w20	w22	w24
1	CH505	CPPP		B		CTF	B	Pw53	B	Pw78	B	Pw100	B	B+kill
2	CH505	CMMP		B		CTF	B	Mw53	B	Mw78	B	Pw100	B	B+kill
3	tHIVconsvX	CM8M		B		C5+C6	B	M3+M4	B		B	M3+M4	B	B+kill
4	tHIVconsvX	CM16M	C5+C6	B	M3+M4		B		B		B	M3+M4	B	B+kill
5	CH505	CPPP				CTF		Pw53		Pw78		Pw100		
	tHIVconsvX	CM8M		B		C5+C6	B	M3+M4	B		B	M3+M4	B	B+kill
	CH505	CPPP				CTF		Pw53		Pw78		Pw100		

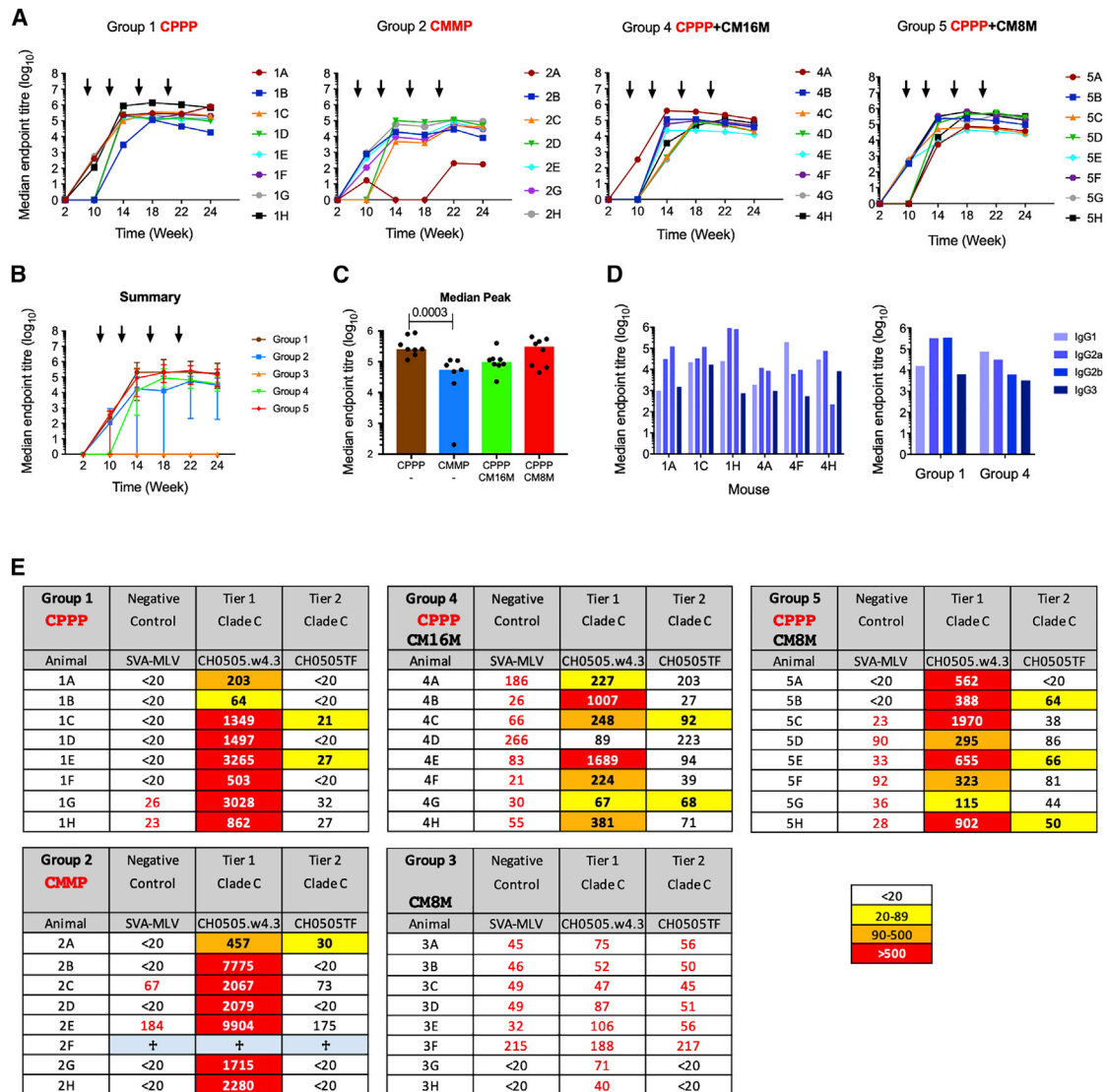
C, recombinant chimpanzee adenovirus; M, recombinant MVA; P, recombinant protein adjuvanted with STR88C; B, drawing a blood sample. Please find vaccine acronyms in Figure 1B.

CH505 regimen CPPP (group 1) outperformed CMMP (group 2) ( $p = 0.0003$ ; Mann-Whitney test). Relative to Ab-vaccine-alone regimen CPPP (group 1), both the temporal separation of the T cell and Ab vaccines (group 4) and their parallel co-administration (group 5) reached lower median titers (adjusted  $p = 0.0518$  and adjusted  $p = 0.6796$ , respectively; Dunn's multiple comparisons test) (Figure 2C). The two main group 4 and 5 comparisons in Mann-Whitney test yielded  $p = 0.3823$ . For three arbitrarily chosen animals in groups 1 and 4, we also investigated induction of the immunoglobulin G1 (IgG1), IgG2a, IgG2b, and IgG3 isotypes and found a good representation of all isotypes and no significant differences between the two tested regimens ( $p = 0.3750$ ; Wilcoxon matched-pairs signed-rank test). Therefore, parallel administration of the tHIVconsvX vaccines did not compromise the CPPP regimen delivering the CH505 EnvSeq-1 trimers for induction of antibodies binding the native-like CH505 w100 Env trimer.

Sera from the final w24 bleed were tested in the TZM-bl cell neutralization assay against the CH505 virus tier-1 variant Ch505.w4.3 and against the tier-2 CH0505TF transmitted-founder virus. There was a good tier-1 virus neutralization compared to that against the control MLV-pseudotyped virus ( $p < 0.0001$ ; ANOVA); however, tier-2 neutralization was not significantly elevated above the murine leukemia virus (SVA-MLV) negative control values of group 3 (Figure 2E). While there was not a statistically significant difference in tier-1 virus neutralization between the CPPP (group 1) and CMMP (group 2) ( $p = 0.1206$ ; Mann-Whitney test), the group mean (1,340 versus 3,757) and median (1,094 versus 2,079) neutralization titers, respectively, were both higher for the latter regimen (Figure 2E). Comparing the CH505 EnvSeq-1 delivery using the CPPP regimen alone (group 1) with temporal separation (group 4) and co-administration (group 5) of the CH505 EnvSeq1 and tHIVconsvX vaccines, there was no significant interference of tier-1 neutralization caused by the T cell vaccine co-administration (adjusted  $p = 0.1218$  and adjusted  $p = 0.5009$ , respectively; Dunn's multiple comparisons test).

**Frequencies of Interferon (IFN)- $\gamma$ -producing tHIVconsvX-specific T Cells Are Not Compromised by Co-administration of the CH505 EnvSeq-1 Vaccines**

All five groups of vaccine recipients were assessed for induction of cellular responses recognizing conserved regions of tHIVconsvX and CH505w100 Env using 10 (P1–P10) and five (PI–PV) pools, respectively, of 15-mer peptides overlapping by 11 amino acids covering the entire immunogens with a particular attention to the two combined regimens. Three groups—3, 4, and 5—receiving the tHIVconsvX vaccines had broadly specific T cell responses, whereby individual mice recognized median (range) of 6 (2–9), 7 (4–9), and 7 (5–8) peptide pools (Figure 3A) and reached total frequencies of 4,404 (354–8,705), 6,062 (475–11,236), and 4,309 (648–8,053) spot-forming units (SFU)/ $10^6$  splenocytes, respectively (Figure 3B). Neither the breadth nor the total tHIVconsvX frequencies were statistically separable among groups 3–5 with  $p = 0.3465$  and  $p = 0.5813$  (Kruskal-Wallis test, ANOVA), respectively. While the median total frequency of the tHIVconsvX-specific T cells induced

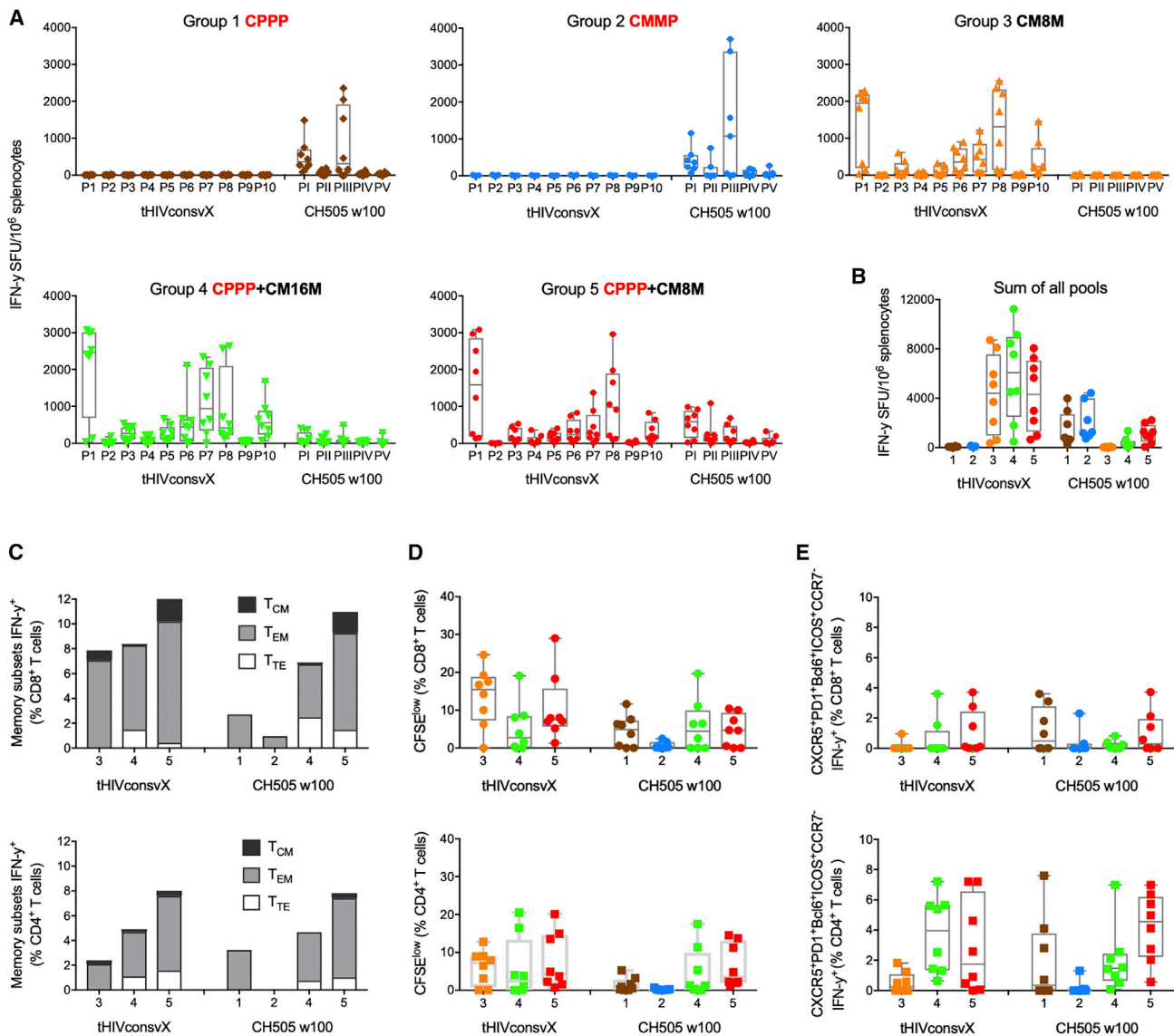


**Figure 2. No Effect on Induction of Antibody Responses with the CH505 EnvSeq-1 Regimen by Co-administration of the tHIVconsvX Vaccines**

Five groups of CD1-SWISS mice were immunized with single (groups 1–3) or B + T cell-combined (groups 4 and 5) regimens as indicated using the following vaccine vectors/modalities: C for ChAdOx1, M for MVA, and P for adjuvanted protein (Table 1) shown in red for the Env and in black for the tHIVconsvX immunogens. (A) Individual mouse endpoint serum titers binding to the Pw100 trimer are shown with the individual vaccine administrations depicted by arrows. Results are shown as average of two wells (n = 8). (B) Summary of the group endpoint binding titers. Results are shown as median (upper and lower limit) (n = 8). (C) The bars show the median peak endpoint titers for each indicated group with individual mouse values given (n = 8). p value obtained using the Mann-Whitney test is shown above. (D) For three mice in groups 1 and 4, the IgG isotypes of the Pw100-binding antibodies were determined and shown for each mouse individually shown as average of two samples (left) or as group medians (n = 3) (right). (E) The TZM-bl cell neutralization assay was used to determine the autologous tier-1 and tier-2 virus neutralization activities. High background non-specific binding was detected (red digits) either against the Moloney leukemia virus-pseudotyped virus or in group 3, which did not receive any Env vaccine. For each animal, its background if > 20 was subtracted from the neutralizing titer before assigning the serum-neutralization activity using the indicated heat scale of white, yellow, orange, and red. Animal 2F died from vaccine-unrelated causes (blue).

by the pre-administration of T cell vaccines prior to CH505 EnvSeq-1 (group 4) was higher relative to the co-administration (group 5), they were again not statistically separable (adjusted p = 0.07159 and adjusted p > 0.9999, respectively; Dunn’s multiple comparisons test). For both combined groups 4 and 5, the frequencies of CH505w100 Env-specific T cells were significantly lower compared

to tHIVconsvX-specific T cells both (p = 0.0006 and p = 0.0281, Mann-Whitney test, respectively), perhaps because of the potential mismatches among the CH505TF, CH505w53, Ch505w78, and Ch505w100 Env epitopes making the boosts less efficient. Possibly for the same reasons, the CMMP regimen was no more immunogenic than CPPP in contrast to what we might have expected for a complex

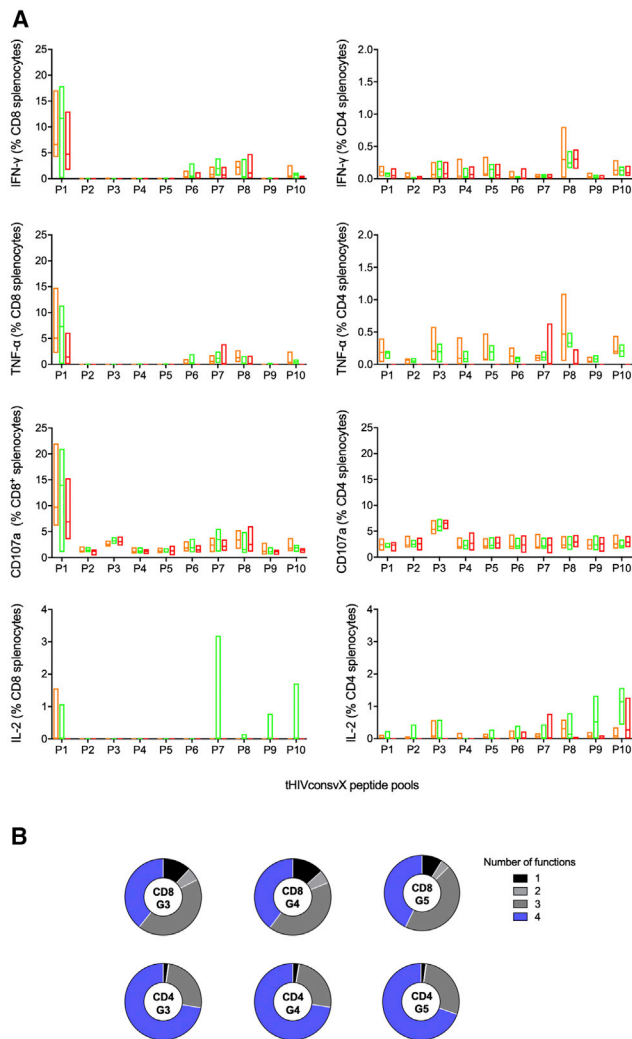


**Figure 3. No Effect on tHIVconsvX-specific T Cell Frequencies, Memory Subtypes, and Proliferation by Co-administration of the CH505 EnvSeq-1 Vaccines**

Groups of CD1-SWISS mice were immunized with an Ab or T cell vaccine alone (groups 1–3) or using Ab+T cell-combined (groups 4 and 5) regimens using the following vaccine vectors/modalities: C for ChAdOx1, M for MVA, and P for adjuvanted protein (Table 1) shown in red for the Env and in black for the tHIVconsvX immunogens. Frequencies of vaccine-elicited T cells were enumerated in a triplicate IFN- $\gamma$  ELISPOT assay using pools of 15/11 peptides derived from tHIVconsvX (P1-P10) and CH505w100 Env (P1-PV) and shown for individual pools (A) or as total responses (B). Results are shown as median (range) per group with individual mouse values shown (n = 8). (C) T cell memory subtypes were defined as T<sub>CM</sub>, central memory (CD44<sup>hi</sup>CD62L<sup>hi</sup>); T<sub>EM</sub>, effector memory (CD44<sup>hi</sup>CD62L<sup>lo</sup>); and T<sub>TE</sub>, terminally differentiated effector T cells (CD44<sup>lo</sup>CD62L<sup>lo</sup>); samples for all relevant groups/peptide of origin combinations were analyzed in duplicates and shown as average (see Figure S2 for gating strategy). *In vitro* proliferative capacity of immune splenocytes producing IFN- $\gamma$  upon specific pooled peptide restimulation was determined in a CFSE proliferation assay (see Figure S3 for gating strategy) (D) and quantified follicular CD3<sup>+</sup>CD8<sup>+</sup> and CD3<sup>+</sup>CD4<sup>+</sup> T cells defined as CXCR5<sup>+</sup>PD-1<sup>+</sup>Bcl6<sup>+</sup>CCR7<sup>-</sup>ICOS<sup>+</sup> and producing IFN- $\gamma$  upon specific peptide stimulation (see Figure S4 for gating strategy) (E). For (D) and (E), data are shown as median + IQR (box) with individual mouse values (n = 8). All data in the figure are shown after subtracting the background peptide-unstimulated control values.

poxvirus vector. Both the CD8<sup>+</sup> and CD4<sup>+</sup> T cells against both immunogens had structured memory phenotypes dominated by effector memory cells (CD44<sup>hi</sup>CD62L<sup>lo</sup>) and central memory and starting development of central memory (CD44<sup>hi</sup>CD62L<sup>hi</sup>) (Figure 3C) and proliferated to specific stimuli (Figure 3D). We also investigated in-

duction of CH505w100 Env-specific follicular T helper (T<sub>fh</sub>) cells defined as peptide-responsive IFN- $\gamma$ <sup>+</sup>CD3<sup>+</sup>CD4<sup>+</sup>CXCR5<sup>+</sup>PD1<sup>+</sup>Bcl6<sup>+</sup>ICOS<sup>+</sup>CCR7<sup>-</sup>. For T cells recognizing the CH505w100 Env, median (range) percentages of CD4<sup>+</sup> T<sub>fh</sub> cells for groups 1, 2, 4, and 5 reached percentages of 0.35 (0–7.6), 0 (0–1.3), 1.48 (0.07–6.98), and



**Figure 4. No Effect on the tHIVconsvX-specific T Cell Functionality by Co-administration of the CH505 EnvSeq-1 Vaccines**

Using the same experimental design as in Figure 3 and Table 1, the CD8<sup>+</sup> and CD4<sup>+</sup> tHIVconsvX vaccine-elicited T cells were assessed for four effector functions, namely IFN- $\gamma$ , TNF- $\alpha$ , and IL-2 production and degranulation by surface expression of CD107a, in an ICS assay. (A) Frequencies of mono-functional specific T cells were compared for CM8M alone (group 3; orange), pre-Env expansion of conserved region-specific T cells in CM16M+CPPP (group 4; green), and parallel T+B cell vaccine delivery in CM8M+CPPP (group 5; red). (B) A multifunctionality of individual cells was determined, and the data are expressed as a proportion of tHIVconsvX-specific CD8<sup>+</sup> or CD4<sup>+</sup> T cells capable of performing 1, 2, 3, or 4 functions. All data in the figure are shown after subtracting the background peptide-unstimulated control values.

4.55 (0.57–6.98), respectively ( $p = 0.0072$ , ANOVA). Although median percentages of CD4<sup>+</sup> Tfh were higher for CPPP (group 1) versus CMMP (group 2) and co-administration (group 5) versus pre-administration (group 4) of the tHIVconsvX vaccines with Ch505 EnvSeq1, these differences were not significant ( $p = 0.1841$  for both, Mann-Whitney test) (Figure 3E). Also marginal induction of follicular

CD8<sup>+</sup> T cells was noted, which might be an important feature of effective anti-HIV-1 responses (Figure 3E). The tHIVconsvX vaccines elicited comparable CD4<sup>+</sup> and low CD8<sup>+</sup> Tfh cells for groups 3, 4, and 5, too. Finally, we assessed the functionality of the tHIVconsvX-specific CD8<sup>+</sup> and CD4<sup>+</sup> T cells induced by the tHIVconsvX vaccines alone or in a combination with the CH505 EnvSeq-1 vaccination as for expression of IFN- $\gamma$ , tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin-2 (IL-2), and CD107a, the degranulation marker. There were no significant differences between the regimens for frequencies of monofunctional cells (Figure 4A) nor for the plurifunctionality (Figure 4B).

Overall, co-delivery of the CH505 EnvSeq-1 vaccines did not significantly affect the induction CD8<sup>+</sup> T cells focused on the conserved regions of the HIV-1 proteome but elicited Env-specific CD4<sup>+</sup> Tfh cells thought to be beneficial for an efficient bnAb induction.

## DISCUSSION

The overarching aim of this work was to study combination regimens of Ab and T cell vaccines. The present study tested two combined vaccination protocols. One aimed at expanding and focusing T cells on the conserved regions of HIV-1 prior to immunization with Env trimers, and the other delivered both the tHIVconsvX and Env trimers derived from four-sequential isolates in parallel. Based on our previous results,<sup>51</sup> we delivered the priming founder-transmitted Env of the CH505 EnvSeq-1 using simian adenovirus ChAdOx1 and boosted with three subsequent protein trimer administrations (PPP). T cells were induced using the in-human-proven regimen of ChAdOx1 prime-two MVA boost. Overall, the CPPP regimen delivering CH505 EnvSeq1 was not affected by prior (group 4) nor parallel (group 5) vaccination with the tHIVconsvX vaccines, and the induced antibody endpoint titers for both binding native-like trimers and HIV-1 tier-1 neutralization remained uncompromised. Similarly, the breadth and total magnitude of the T cell responses recognizing conserved regions of HIV-1 elicited by the two combined regimens were not statistically separable from the CM8M regimen delivering tHIVconsvX alone (group 3). For both combined regimens, significantly higher frequencies of T cells were elicited recognizing tHIV-consvX than the CH505 Env.

The CPPP (group 1) and CMMP (group 2) regimens were used as comparators for antibody-alone induction, which delivered the four sequential CH505 Env isolates as SOSIP.6R.664 trimers using different vectors. In our previous rabbit immunizations using BG505 SOSIP.6R.664 trimers, C provided the best prime over P.<sup>51</sup> This was not the case in this study. Here, we did not use PTF as the prime, so it is not clear whether or not the superiority of C over P prime would hold in this experimental setting. Also, CMMP underperformed relative to CPPP in terms of binding antibody titers (Figure 2C) and induction of follicular CD4<sup>+</sup> T cells (Figure 3E). The most obvious differences between the past and present work are the use of rabbits with multiple repetitive primes with stabilized CH505 TF SOSIP timer in prior work<sup>30</sup> versus an outbred mouse stock as the animal model and homologous versus time-isolate heterologous Env immunization due to the lineage immunization, respectively.

For all regimen groups, it took two trimer administrations to reach the plateau levels of binding antibody titers, which were maintained rather than further increased by the last two vaccinations. This is desirable, as the strongly expanded Ab responses induced by the first two CTF and Pw53 vaccines might be matured by the two subsequent Pw78 and Pw100 vaccinations toward the desired bnAb specificity. For group 4, exploring the pre-Env expansion of T cells, the C antibody prime was much less efficient, presumably because of the pre-existing immunity to the ChAdOx1 vector induced by the T cell vaccines (Figures 2A and 2B). Finally, Pw100 was used as the ELISA antigen for all time points, and it can be only estimated how different the results would be if we used PFT, Pw53, Pw78, and Pw100 after the first, second, third, and fourth immunizations, respectively. Also noted is that, in previous rabbit and monkey studies with the CH505 EnvSeq-1 vaccination, animals received three administrations of the PTF prime prior to the Pw53, Pw78, and Pw100 boosts.<sup>30</sup>

HIV-1 neutralization achieved by the CH505 EnvSeq-1 vaccination was determined in the TZM-bl cell assay for the tier-1 CH0505.w3.4 variant and tier-2 CH0505 transmitted-founder virus. As it is often the case with mouse sera, background activity was detected in most of the samples. We did detect neutralization of the tier-1 CH0505.w4.3 virus in most samples, but some sera in tHIVconsvX-only group 3 also gave weak signals against the tier-2 virus, suggesting a possible contribution of non-specific activity to the neutralization signal in the other samples. Very few samples neutralized tier-2 virus CH505TF (Figure 2E), which is a limitation of this study perhaps due to the use of murine model. Thus, while murine B cell receptor repertoire does not constitute the optimal model for fine tuning of the bnAb induction, the primary aim of the present study was to assess the effect of combining Ab and T cell vaccines, and from that point, neutralization in the combined groups was not significantly inferior to Env immunization alone, with the caveat that groups of eight animals were too small for outbred mice to reveal subtle differences.

The frequencies and quality of the tHIVconsvX-specific T cells was not decreased by co-administration of the CH505 EnvSeq-1 antibody lineage vaccines. While the CH505 Env trimers did elicit Env-specific T cell responses, these were of lower frequencies and were not induced at the expense of those recognizing the protective conserved regions, perhaps reflecting that the boosting with variant epitopes of EnvSeq-1 was less efficient. The cells were plurifunctional, and 4 weeks after immunization the effector memory T cell ( $T_{EM}$ ) subtype dominated for both  $CD8^+$  and  $CD4^+$  T cells. This outcome is possibly the best we could have hoped for T cells: unaffected tHIVconsvX T cell specificities expanded in breadth by lower levels of T cells recognizing Env (which remain non-protective). Our results also suggest that the B cell ontogeny strategy for induction of bnAb might be more suitable for combining with T cell vaccines than the epitope-based approach, because of the less-efficient Env-specific T cell induction by heterologous immunogens.

Both the T and B cell vaccine regimens induced follicular  $CD4^+$  and  $CD8^+$  T cells. The lymphoid follicle is central to the development of

humoral immune responses, and cell entry into this site is highly regulated, which likely contributes to the establishment of viral reservoirs in these sites. Follicular  $CD8^+$  T cells may play an important role targeting HIV-1-infected  $CD4^+$  Tfh cells as well as in the Tfh-cell regulation.<sup>66,67</sup> Thus, it was encouraging to see at least in some animals that follicular  $CD8^+$  T cells were induced. The frequencies of tHIVconsvX-specific  $CD4^+$  Tfh cells were higher for the combined CPPP/CM16M regimen (group 4) relative to the CM8M alone (group 3).

In conclusion, encouraging initial results were obtained for combining two promising vaccine strategies, one eliciting T cell responses and one employing Env lineage immunization, into one protocol. The lineage immunization is particularly suitable for its less-efficient induction of Env-specific T cells and could be likely further improved by using protein-only modality for the EnvSeq-1 immunogens.

## MATERIALS AND METHODS

### Construction of the ChAdOx1-Vectored CH505 EnvSeq-1 SOSIP Vaccines

Vaccine vector ChAdOx1 is derived from simian (chimpanzee) adenovirus isolate Y25 of adenovirus group E.<sup>52</sup> For the generation of recombinant ChAdOx1, the DNA fragments carrying open reading frames (ORFs) of the individual sequential, modified CH505 Env gp140 isolates, designated as EnvSeq-1,<sup>58</sup> were inserted into the E1 locus of the adenovirus genome under the control of the human cytomegalovirus immediate-early promoter, while the adenovirus genome was stably integrated in a bacterial artificial chromosome. The recombinant ChAdOx1 vaccines were rescued by transfection of purified excised genomic DNA into HEK293A T-REX (tetrayclin-sensitive repressor of transgene expression used for preparation of recombinant simian adenoviruses) cells (Thermo Fisher). The transgene presence and absence of contaminating empty parental adenovirus in the virus stocks of ChAdOx1.tCH505TF, ChAdOx1.tCH505w53, ChAdOx1.tCH505w78, and ChAdOx1.tCH505w100 were confirmed by PCR, and the viruses were titered and stored at  $-80^{\circ}\text{C}$  until use.

### Construction of the MVA-vectored CH505 EnvSeq-1 SOSIP Vaccines

To generate recombinant MVA vaccines, the DNA fragments coding for the individual sequential isolates of the CH505 Env gp140<sup>58</sup> were inserted directly into the MVA genome by homologous recombination in chicken embryo fibroblast cells. The expression cassettes were directed under the control of the modified H5 poxvirus promoter and into the thymidine kinase locus of the MVA genome. Initially co-inserted EGFP marker was removed by trans-dominant recombination to generate markerless vaccines MVA.tCH505w53, MVA.tCH505w78, and MVA.tCH505w100.<sup>13</sup> The virus was plaque purified, expanded, purified on a 36% sucrose cushion, tittered, and stored at  $-80^{\circ}\text{C}$  until use.

### Production of the CH505 EnvSeq-1 SOSIP Protein Trimer Vaccines

CH505w053.16chim.6R.SOSIP.664v4.1/293F (lot 303ESD), CH505w078.33chim.6R.SOSIP.664v4.1/293F (lot 591HC), and CH505w100.16B6chim.6R.SOSIP.664v4.1/293F (lot 635HC) trimer proteins were



produced and purified as reported previously.<sup>58,68,69</sup> In brief, the protein trimers were expressed in HEK293T cells to have a human cell glycosylation profile by transient transfection of DNA together with a co-transfected Furin gene to boost the level of cleavage and purified using a 3B3 mAb<sup>70</sup> affinity column followed by size-exclusion chromatography and stored at  $-80^{\circ}\text{C}$  until use.

The quality control of each protein preparation was assessed.<sup>30</sup> Each of the trimer preparations was analyzed by biolayer interferometry to obtain an antigenic profile for neutralizing and non-neutralizing antibodies. To confirm the size of trimer gp140, 50  $\mu\text{g}$  of each trimer preparation was fractionated using Superose 6 chromatography, and 1  $\mu\text{g}$  of each protein was fractionated by blue native PAGE. Lastly, greater than 10,000 trimers per preparation were visually inspected for formation of envelope trimers by negative stain electron microscopy, and the percentages of particles that were native-like envelope trimers were calculated.

#### Construction of the tHIVconsvX Vaccines

The construction and preparation of the ChAdOx1.tHIVconsv5&6 and MVA.tHIVconsv3&4 vaccines were published previously<sup>13</sup> and were similar to the procedures described for the CH505 EnvSeq-1 vaccines above.

#### SDS-PAGE and Immunogen Expression by Western Blot

Human HeLa cells were infected with recombinant ChAdOx1 at MOI 10 or MVA (MOI 5) vaccines and lysed 48 h later in the presence of protease inhibitors. Individual polypeptides of the cell lysates were separated on SDS-polyacrylamide gels cross-linked with 15% (*N,N*-diallyltartardiamide [DATD]) using Novex Nu-PAGE SDS-PAGE system (Life Technologies) electrophoresis system. Separated polypeptides of one gel were transferred onto a nitrocellulose filter (Amersham International) using the transfer blot turbo system (Bio-Rad) and a second gel was stained with Coomassie blue. The primary 3B3 mAb<sup>70</sup> and anti-mouse IgG secondary antibody conjugated to horseradish peroxidase (HRP) (715-035-150, Jackson ImmunoResearch Laboratories) incubations and wash steps were carried out using the iBIND Western system (Life Technologies). The dilution of both the antibodies was 1:1,000. Bound antibodies were visualized by enhanced chemiluminescence (ECL; Amersham International).

#### Peptides

15-mer peptides overlapping by 11 amino acids (15/11) spanning the entire CH505 w100 gp140 ORF were obtained from Pepscan (the Netherlands) and combined into pools designated PI–PV, and two ORFs of tHIVconsvX mosaic 1 and mosaic 2 were made at Ana Spec (USA) and combined into pools P1–P10. Peptides were dissolved in DMSO at a concentration of 20 mg/mL and stored at  $-80^{\circ}\text{C}$ . Working stocks of 4 mg/mL were prepared by diluting 20-mg/mL stocks with PBS. Peptides were used in assays at a final concentration of 2  $\mu\text{g}/\text{mL}$  per peptide.

#### Power Calculation for Group Size

We refer to this experiment as a pilot, hypothesis-generation study. To our knowledge, for antibody induction, this is the first time that

the CH505 EnvSeq1 immunogens, especially delivered by combination of C, M, and P, were tested in outbred CD1-SWISS mouse stock and, therefore, any estimation of the binding reciprocal titers and tier-1/2 neutralization would have to be based on rabbit or monkey studies<sup>30</sup> or on the uncleaved BG505 SOSIP immunization of the BALB/c mice.<sup>28</sup> For T cell induction, the closest experiment in CD1-SWISS mice using the ChAdOx1.tHIVconsv5&6-MVA.tHIVconsv3&4 vaccines in regimen CM (not CMM) was published by Wee et al. 2016.<sup>62</sup> In this experiment, 7 CD1-SWISS responded by average total responses to pools P1–P10 of median (range) 3,730 (0–8,600) SFU/ $10^6$  splenocytes; therefore, we would need 1,926, 693, and 98 mice per group to detect a 10%, 25%, and 50% difference between two equally sized groups. The median (range) breadth of responses in terms of number of recognized pools out of 10 was 3 (0–7). Here, we would need 1,056, 1,529, and 2,389 mice per group to detect a significant difference of 1, 2, and 3 pools between two equal groups of mice. These are the minimum numbers of animals needed to attain statistical significance of  $p < 0.05$  with an 80% probability (<http://research.illinois.edu/regulatory-compliance-safety/iacuc>). Thus, for a pilot study, we consider groups of eight animals adequate.

#### Mice and Immunizations and Preparation of Splenocytes

Six-week-old female CD1-SWISS mice were purchased from Charles River (UK) and housed at the Functional Genomics Facility, University of Oxford. Mice were immunized intramuscularly under general anesthesia either with  $10^8$  infectious units (IU) of rChAdOx1s,  $5 \times 10^6$  plaque-forming units (PFU) of rMVAs, or 30  $\mu\text{g}$  of protein adjuvanted with 10% STR8sC (0.2 mg/mL oligodeoxynucleotides [ODNs]; 0.5 mg/mL R848) (Table 1). On the day of sacrifice, spleens were collected and cells isolated by pressing organs individually through a 70- $\mu\text{m}$  nylon mesh of a sterile cell strainer (Fisher Scientific) using a 5 mL syringe rubber plunger. Following the removal of red blood cells with RBC lysing buffer hybri-max (Sigma), splenocytes were washed and re-suspended in R10 (RPMI 1640 supplemented with 10% FCS, penicillin/streptomycin and  $\beta$ -mercaptoethanol) for ELISPOT, intracellular cytokine staining (ICS) assays, and other procedures.

#### Ethics Statement

All animal 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.

#### The IFN- $\gamma$ ELISPOT Assay

The ELISPOT assay was performed using the mouse IFN- $\gamma$  ELISPOT kit (Mabtech) according to the manufacturer's instructions. Immune splenocytes were collected and tested separately from individual mice. Peptides were used at 2  $\mu\text{g}/\text{mL}$  each, and splenocytes at  $2 \times 10^5$  cells/well were added to 96-well high-protein-binding Immobilon-P membrane plates (Millipore) that had been precoated with 5  $\mu\text{g}/\text{mL}$  anti-IFN- $\gamma$  monoclonal antibody (mAb) AN18 (Mabtech, Stockholm, Sweden). The plates were incubated at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$  for 18 h

and washed with PBS before the addition of 1  $\mu\text{g}/\text{mL}$  biotinylated anti-IFN- $\gamma$  Mab (Mabtech) at room temperature for 2 h. The plates were then washed with PBS, incubated with 1  $\mu\text{g}/\text{mL}$  streptavidin-conjugated alkaline phosphatase (Mabtech) at room temperature for 1 h, and washed with PBS, and individual cytokine-producing units were detected as dark spots after a 10-min reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitrobluetetrazolium using an alkaline phosphatase-conjugate substrate (Bio-Rad, Richmond, CA, USA). SFU were counted using the AID ELISpot reader system (Autoimmun Diagnostika). The frequencies of responding cells were expressed as a number of SFU/ $10^6$  splenocytes after subtracting the background values.

#### IFN- $\gamma$ Capture and Memory T Cell Subtype Assay

Splenocytes were stimulated with a mix of peptide pools P1+P8 for tHIVconsvX and PI+PIII for CH505 at 2  $\mu\text{g}/\text{mL}$  per peptide or a tissue culture medium with 1% DMSO as a negative control. The cells were washed with PBS (pH 7.2) plus 0.5% BSA and 2 mM EDTA, labeled with 10  $\mu\text{L}$  of IFN- $\gamma$ -catch reagent for 5 min at 4°C followed by the addition of 1 mL of warm media and incubated at 37°C for 45 min on a tube rotator (VWR). Immediately following incubation, cells were placed on ice for 5 min, washed, and stained at 4°C for 10 min with 100  $\mu\text{L}$  of a mastermix containing anti-IFN- $\gamma$  phycoerythrin (PE) mAb (Miltenyi Biotec) with LIVE/DEAD fixable aqua stain (Molecular Probes, Invitrogen) and the anti-membrane marker mAbs CD3 allophycocyanin (APC), CD4 fluorescein isothiocyanate (FITC), CD8a eFluor 450, CD44 Alexa Fluor 700, and CD62-L PE-Cy7 (all from Thermo Fisher Scientific). Cells were washed and fixed with 1% paraformaldehyde in PBS prior to running on an LSRII flow cytometer (Becton Dickinson). The frequencies of the subtypes in CD8<sup>+</sup> and CD4<sup>+</sup> T cells represent the differences in stimulated and unstimulated immune cells. All data are shown after subtracting the background values.

#### ICS Assay

Splenocytes were stimulated with 2  $\mu\text{g}/\text{mL}$  of peptide pools described in the above assay, ionomycin, and phorbol myristate acetate (PMA) at 2.0  $\mu\text{g}/\text{mL}$  and 0.5  $\mu\text{g}/\text{mL}$ , respectively, or a tissue culture medium with 1% DMSO as a negative control. The cultures were supplemented with anti-CD107a PE-conjugated mAb (Thermo Fisher Scientific). The cells were incubated at 37°C, 5% CO<sub>2</sub> for 2 h prior to the addition of brefeldin A and monensin (BD Biosciences) and then left in culture overnight. The cells were centrifuged briefly and washed in PBS plus 5% BSA (Sigma-Aldrich), and the pellet was resuspended in 40  $\mu\text{L}$  of anti-CD16/32 mAb block with LIVE/DEAD fixable aqua stain (Molecular Probes, Invitrogen). Cells were washed, a mastermix of anti-membrane marker mAbs was prepared containing CD4 APC/Cy7 (BioLegend), CD3 PerCP-eFluor710, and CD8a eFluor 450 (both from Thermo Fisher Scientific), and 40  $\mu\text{L}$  was added to each tube. The cells then were incubated at 4°C for 30 min, permeabilized using fix-perm solution (Becton Dickinson) at 4°C for 20 min, washed with perm wash buffer (Becton Dickinson), and incubated with a prepared mastermix containing anti-IFN- $\gamma$  PE-Cy7, anti-IL-2 APC, and anti-TNF- $\alpha$  FITC mAbs (all from

Thermo Fisher Scientific), incubated at 4°C for 30 min, washed, and resuspended in perm wash buffer prior to running on an LSRII flow cytometer (Becton Dickinson). All data represent background-subtracted values.

#### CSFE Proliferation Assay

Splenocytes were resuspended in pre-warmed PBS with 0.1% BSA at a final concentration of  $1 \times 10^6$  cells/mL and labeled with 750 nM CFSE for 10 min at 37°C, 5% CO<sub>2</sub>. The staining was quenched by adding 5 volumes of ice-cold R10 followed by a 5-min incubation on ice. The cells were pelleted, washed, and plated in 96-well round-bottom plates at a concentration of  $1 \times 10^6$  cells/well. The CFSE-labeled cells were then stimulated for 5 days with 2  $\mu\text{g}/\text{mL}$  of peptide pools described in the memory phenotypic assay, 2.0  $\mu\text{g}/\text{mL}$  ionomycin, and 0.5  $\mu\text{g}/\text{mL}$  PMA (positive control), or tissue culture media with 1% DMSO (negative control). The cells were stained with a mastermix containing the dead cell marker (LIVE/DEAD fixable aqua stain; Invitrogen) and anti-membrane marker mAbs anti-CD4-APC/Cy7 (BioLegend), anti-CD3-PerCPeFluor710, and anti-CD8-eFluor450 (both from eBioscience), fixed, and acquired on a BD LSR II flow cytometer (Becton Dickinson). All data are shown as background-subtracted values.

#### Phenotyping Vaccine-Elicited Follicular T Cells

Splenocytes were stimulated with peptide pools P1+P8 for tHIVconsvX and PI+PIII for CH505 at 2  $\mu\text{g}/\text{mL}$  per peptide and supplemented with anti-CD28 and anti-CD49d mAbs (Thermo Fisher Scientific) both at 1.0  $\mu\text{g}/\text{mL}$ , or a tissue culture medium supplemented with 1% DMSO and anti-CD28 and anti-CD49d mAbs, but no added peptides as a negative control. The cells were incubated at 37°C, 5% CO<sub>2</sub> for 2 h prior to the addition of brefeldin A and monensin (BD Biosciences) and then left in culture overnight. The cells were centrifuged briefly and washed in PBS plus 5% BSA (Sigma-Aldrich), and the pellet re-suspended in 40  $\mu\text{L}$  of CD16/32 with LIVE/DEAD fixable aqua stain (Molecular Probes, Invitrogen). Cells were washed, a mastermix of anti-membrane marker mAbs was prepared containing CD4 APC/Cy7 (BioLegend), CD19 FITC, CD14 FITC, CD16 FITC, CD3 PerCP-eFluor710, CD8a eFluor 450, CD44 BV605, CCR7 Alexa Fluor 700, CXCR5 PE/Cy7, and PD-1 PerCP-eFluor610 (all from Thermo Fisher Scientific), and 40  $\mu\text{L}$  was added to each tube. The cells were incubated at 4°C for 30 min and then permeabilized using fix-perm solution (BD Biosciences) for 20 min at 4°C. The cells were washed with perm wash buffer (BD Biosciences), and a mastermix of anti-cytokine and anti-transcription factor mAbs was prepared containing BCL6 PE and IFN- $\gamma$  APC (Thermo Fisher Scientific). The cells were incubated at 4°C for 30 min, washed, and resuspended in perm wash buffer prior to running on an LSRII flow cytometer (Becton Dickinson). All antibodies were used at pre-titrated, optimal concentrations, and the data are presented after subtracting the background.

#### Capture ELISA for Quantifying Binding Abs in Murine Sera and Isotyping

Human mAb PGT151-4A at 2.0  $\mu\text{g}/\text{mL}$  in bicarbonate buffer (0.1 M sodium bicarbonate; Sigma) was coated onto ELISA plates

(Greiner Bio-One) at 4°C during an overnight incubation. The wells were washed 3× in PBS-T (PBS, 0.05% Tween 20) and blocked with SuperBlock (4% whey protein, 15% normal goat serum, and 0.05% Tween 20 in PBS). After washing 3× with PBS-T, the CH505w100 trimer at 0.2 µg/mL in SuperBlock was added to all wells at room temperature (RT) for 2 h. After washing 3× in PBS-T, serial 5-fold dilutions of sera in SuperBlock starting from 1:100 were added to wells at RT for 2 h; the plates were washed 5× in PBS-T, incubated with 50 µL/well of secondary anti-mouse IgG HRP-conjugated antibody at 0.05 µg/mL (715-035-150, Jackson ImmunoResearch Laboratories) in Superblock at RT for 1 h, and washed 3× with PBS-T. Alternatively, for IgG isotype determination, biotinylated secondary anti-mouse IgG1, IgG2a, IgG2b, and IgG3 mAbs were used (BD Biosciences 553441, 553388, 553393, and 553401, respectively), diluted to 1 µg/mL in PBS-T. The colorimetric reaction was carried out using the 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase substrate for 10 min, the reaction was stopped by addition of 50 µL of 0.33 M HCl, and the plates were read immediately at OD<sub>450</sub>.

#### HIV-1-Neutralization Assay

A validated TZM-bl neutralization assay using Env-pseudotyped viruses was described previously.<sup>71</sup> The sera were tested against the tier-2 CH0505TF transmitted-founder virus and against tier-1 variant of this virus CH0505.w4.3 for neutralizing activity. Activity against Moloney leukemia virus-pseudotyped virus was used to estimate the HIV-1 non-specific activity in the assay. For additional information on the assay and related protocols, see <https://www.hiv.lanl.gov/content/nab-reference-strains/html/home.htm>.

#### Statistical Analysis

Statistical analyses were performed using Prism v7 (GraphPad Software). ELISPOT and flow cytometry results were assumed to be non-Gaussian in distribution; thus, non-parametric tests were used throughout and medians (range) are shown. Two-group comparisons were performed with Mann-Whitney test. Multi-group comparisons were performed with Kruskal-Wallis test and Dunn's multiple comparison post-test. Correlation between neutralization of different viruses was analyzed by using the Spearman non-parametric method. A *P* value (two-tailed) < 0.05 was considered significant.

#### SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.omtm.2019.06.003>.

#### AUTHOR CONTRIBUTIONS

T.H. conceived the work; T.H., E.G.W., F.D., D.C.M., N.A.M., N.B., B.F.H., and C.L. designed and/or carried out the experiments; K.O.S. and B.F.H. provided essential reagents; T.H. and B.F.H. raised the funds; T.H. wrote and all authors critically reviewed the manuscript.

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