Published in final edited form as:

Science. 2015 August 14; 349(6249): aab1253. doi:10.1126/science.aab1253.

Diversion of HIV-1 Vaccine-induced Immunity by gp41-Microbiota Cross-reactive Antibodies

Wilton B Williams^{*,1}, Hua-Xin Liao¹, M. Anthony Moody¹, Thomas B. Kepler³, S Munir Alam¹, Feng Gao¹, Kevin Wiehe¹, Ashley M. Trama¹, Kathryn Jones¹, Ruijun Zhang¹, Hongshuo Song¹, Dawn J Marshall¹, John F Whitesides¹, Kaitlin Sawatzki³, Axin Hua³, Pinghuang Liu¹, Matthew Z Tay¹, Kelly Seaton¹, Xiaoying Shen¹, Andrew Foulger¹, Krissey E. Lloyd¹, Robert Parks¹, Justin Pollara¹, Guido Ferrari¹, Jae-Sung Yu¹, Nathan Vandergrift¹, David C. Montefiori¹, Magdalena E Sobieszczyk⁷, Scott Hammer⁷, Shelly Karuna⁵, Peter Gilbert⁴, Doug Grove⁴, Nicole Grunenberg⁵, Julie McElrath⁵, John R. Mascola², Richard A Koup², Lawrence Corey⁵, Gary J. Nabel^{2,Φ}, Cecilia Morgan⁴, Gavin Churchyard⁸, Janine Maenza⁵, Michael Keefer⁶, Barney S. Graham², Lindsey R. Baden⁹, Georgia D. Tomaras¹, and Barton F. Haynes^{*,1}

¹Duke Human Vaccine Institute, Duke University School of Medicine, Durham, NC

²Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institute of Health, Bethesda, MD

³Department of Microbiology, Boston University School of Medicine, Boston, MA

⁴SCHARP, Fred Hutchinson Cancer Research Center, Seattle, WA

⁵Vaccine and Infectious Disease Division, Fred Hutchinson Cancer Research Center, Seattle, WA

⁶University of Rochester School of Medicine, Rochester, NY

⁷Department of Medicine, Columbia University Medical Center, New York, NY

⁸The Aurum Institute, Johannesburg, SA

⁹Brigham and Women's Hospital, Boston, MA

Abstract

A HIV-1 DNA prime-recombinant Adenovirus Type 5 (rAd5) boost vaccine failed to protect from HIV-1 acquisition. We studied the nature of the vaccine-induced antibody (Ab) response to HIV-1 envelope (Env). HIV-1-reactive plasma Ab titers were higher to Env gp41 than gp120, and repertoire analysis demonstrated that 93% of HIV-1-reactive Abs from memory B cells was to Env gp41. Vaccine-induced gp41-reactive monoclonal antibodies (mAbs) were non-neutralizing, and frequently polyreactive with host and environmental antigens including intestinal microbiota (IM). Next generation sequencing of an IGHV repertoire prior to vaccination revealed an Env-IM

^{*}Correspondence to: Wilton B. Williams, Ph.D. and Barton F. Haynes, M.D., 2 Genome Court, MSRBII Bldg. Room 4090, DUMC 103020, Duke University Medical Center, Durham, NC 27710, Ph: 919-684-5279 Fx: 919-684-5230, barton.haynes@duke.edu; wilton.williams@duke.edu.

^ΦCurrent address: Sanofi, 640 Memorial Drive, Cambridge, MA, 02139.

SUPPLEMENTARY MATERIALS

Tables S1–S25, Figures S1–S12

cross-reactive Ab that was clonally-related to a subsequent vaccine-induced gp41-reactive Ab. Thus, HIV-1 Env DNA-rAd5 vaccine induced a dominant IM-polyreactive, non-neutralizing gp41-reactive Ab repertoire response that was associated with no vaccine efficacy.

In acute HIV-1 infection, the dominant initial plasma antibody (Ab) response is to the gp41 subunit of the envelope (Env) glycoprotein of the virus (1). This antibody response derives from polyreactive B cells that cross-react with Env and intestinal microbiota (IM) (2, 3). However, it is unknown if a similar gp41-reactive Ab response would occur in the setting of HIV-1 Env vaccination. A DNA prime, recombinant adenovirus serotype 5 (rAd5) boost vaccine that included HIV *gag*, *pol* and *nef* genes, as well as a trivalent mixture of clade A, B and C *env* gp140 genes containing both gp120 and gp41 components was studied in the HIV Vaccine Trials Network (HVTN) [phase Ib (HVTN 082), phase II (HVTN 204), phase IIb (HVTN 505) efficacy trial] and other clinical trials [phase I/II (RV172), phase I (V001)] (4–7). This vaccine was the first vaccine containing the ectodomain of the Env gp41 component, covalently linked to gp120, to be tested in an efficacy trial, and was designed to primarily generate CD8 T cell responses, although this vaccine generated Env Ab responses as well (8–10). However, the phase IIb HVTN 505 efficacy trial showed no vaccine efficacy (11). Thus, these vaccine trials containing Env gp41 provided an opportunity to determine if the Env Ab response in the setting of Env vaccination was dominated by gp41-reactive Abs derived from Env-IM cross-reactive B cells.

Isolation of Env-reactive Memory B Cells and Vaccinee Plasma Serologies

We found that the DNA prime, rAd5 boost antibody response to HIV-1 Env was dominantly focused on gp41 compared to gp120. This specificity was demonstrated by both serologic analysis and by vaccine-Env flow cytometry-sorted memory B cells. Plasma IgG binding assays were performed on plasma of a random sample of 40 Phase IIb efficacy trial vaccine recipients who were HIV-1 negative at the final month 24 visit (11) (Figure 1A), and plasma of 8 HIV-1 uninfected Phase Ib and II DNA prime, rAd5 boost trial participants with high titers of plasma binding Abs to recombinant (r)gp140 vaccine-Envs and/or neutralization of clade C MW965 HIV-1 isolate (Figure 1B). Plasma binding gp41-reactive Ab titers were 10 fold higher than gp120-reactive Ab titers, including Ab reactivity with vaccine-gp120s ($p < 0.0001$ (Figure 1A), $p < 0.01$ (Figure 1B); Wilcoxon signed rank test). Thus, the non-protective DNA prime, rAd5 boost gp140 vaccine induced a dominant HIV-1 Env gp41 plasma Ab response.

Next, we performed single memory B cell sorting by flow cytometry using peripheral blood B cells from phase Ib and phase II DNA prime, rAd5-boost trial participants. Vaccine-Env gp140 and V1V2 subunits, as well as a consensus group M gp140 Env (termed CON-S) (12) were used as fluorophore-labeled recombinant proteins to identify Env-specific memory B cells present in peripheral blood mononuclear cells (PBMCs) of vaccinees 4 weeks after final vaccination (Figure S1) (Table S1). We studied 8 phase Ib and phase II DNA prime, rAd5-boost trial participants; from these 8 vaccinees we isolated 221 HIV-1 Env-reactive Abs (Figure 1C, Table S2). Of the 221 HIV-1 Env-reactive Abs, there were 131 unique V_HDJ_H rearrangements (Table S3). Remarkably, 205/221 (93%) of the HIV-1 Env-reactive Abs and 115/131 (88%) of the unique heavy chain sequences induced by the vaccine were

gp41-reactive, with only 7% (16/221) gp120-reactive (Tables S3–6). We used Ab gene transient transfections to perform ELISAs to determine gp41 versus gp120 reactivity (13). Of the Env-reactive Abs, 16/16 (100%) gp120-reactive and 195/205 (95%) gp41-reactive Abs bound vaccine-rgp140 proteins. The 10 gp41-reactive Abs that bound only heterologous recombinant Env proteins likely recognized gp41 epitopes expressed on the vaccine protein generated by DNA or rAd5 that were not expressed on the rgp140 proteins.

We asked if there were indeed fewer gp120-reactive memory B cells from gp140-vaccinated individuals who received the DNA prime, rAd5 boost vaccine compared to gp140-reactive memory B cells. In 3 Phase II trial vaccinee memory B cell samples, we found that VRC-A gp120 bound to 0.37% of memory B cells compared to 0.55% of memory B cells by VRC-A gp140 (p -value<0.001; Cochran-Mantel-Haenszel Test) (Figure S2). Of the gp41-reactive and gp120-reactive Abs isolated from the 8 Phase Ib and II vaccinees, 112/221 (52%) were sorted using the group M consensus CON-S gp140 as a fluorophore-labeled hook. CON-S rgp120 bound to 0.05% memory B cells in comparison to 0.28% by CON-S gp140 (p -value<0.001, Cochran-Mantel-Haenszel Test). Of total HIV-1 Env-reactive memory B cells, CON-S gp120 bound to 13% (38/292) whereas 87% of Env-reactive memory B cells (254/292) reacted with CON-S gp140 (Figure S2). Therefore, the dearth of isolated gp120-reactive Abs was mirrored by low frequencies of gp120-memory B cells in the blood of vaccinees.

For comparison, we have previously studied an Env gp120-only immunization trial in humans and demonstrated that at peak immunization the frequency of memory B cells ranged as high as 0.73% gp120-reactive B cells (mean $0.23 \pm 0.1\%$) (14). In a rhesus macaque immunization study with gp120 alone, the frequency of memory B cells that were gp120-reactive was 3% (15). Thus, in the setting of gp120-only immunizations in humans and rhesus macaques, the memory B cell response to gp120 is robust.

Heavy chain gene restriction for HIV-1 gp41-reactive memory B cell Abs

We analyzed the variable heavy gene families used by HIV-1-reactive Abs induced by the DNA prime, rAd5 boost gp140 vaccine. Gp41-reactive Abs had a mean heavy chain nucleotide mutation frequency of 2.5% (Figure S3), and a median heavy chain complementary determining region-3 (HCDR3) length of 14 amino acids (Figure S4). Remarkably, of 137 total IGHV1-using Abs, 125 (91% of IGHV1-using Abs, 57% of total 221 HIV-1-reactive Abs) used a single V_H segment, IGHV1–69 (Table S7). In order to rule out IGHV1–69 predominance as a result of PCR primer bias, we examined the heavy chain gene usage of non-HIV-1-reactive Abs isolated from all 8 vaccinees; only 12% (18/145) of non-HIV-1-reactive Abs used IGHV1–69 (p <0.0001, Fisher's exact test). The 125 IGHV1–69-using HIV-1-reactive Abs all reacted with gp41, and 94 (75%) were found to be naturally paired with a kappa light chain and 31 (25%) with a lambda light chain. IGκV3–20 (48%, 60/94) and IGλ2–14 (29%, 9/31) were the light chains preferentially paired with the IGHV1–69-using gp41-reactive Abs (Table S8). Sixty-six percent (136 of 205 Abs) of gp41-reactive Abs used IGHV1 gene family; 61% (125 of 205 gp41-reactive Abs) specifically utilized IGHV1–69 gene segment (Table S9), compared to HIV-1-uninfected individuals where 6% of the Ab repertoire utilized IGHV1–69 (16) (p <0.0001, Chi square test). One

hundred and thirty-two gp41-reactive Abs paired with IG κ V light chains (Table S10), while 73 gp41-reactive Abs paired with IG λ V light chains (Table S11). Moreover, next generation sequencing (NGS) of pre-vaccination heavy chain V_HDJ_H rearrangements derived from RNA obtained from PBMC of all 8 vaccinees revealed ~5% IGHV1–69-using B cells prior to vaccination (Tables S12). Thus, the frequency of IGHV1–69-using B cells was the same in healthy subjects as in pre-vaccination B cells, but was dominantly used in the gp41 post-vaccination repertoire, indicating that the post-vaccination gp41-reactive Ab IGHV1–69 usage induced by the DNA prime, rAd5 boost vaccine was not due to heavy chain gene primer bias, but rather was selected by the vaccine.

There are 13 known allelic variants of IGHV1–69 gene segment; 7 have a phenylalanine (F) at amino acid position 54 in the HCDR2 region (54F-HCDR2), and 6 have a leucine (L) (54L-HCDR2) (17). The ratio of 54F-HCDR2 to 54L-HCDR2 IGHV1–69 allelic variants in the global population is estimated to be 3:2 (18). Neutralizing influenza and HIV-1-reactive Abs use 54F-HCDR2 IGHV1–69 gene segments to bind hydrophobic pockets in the stems of hemagglutinin (HA) (19, 20) and gp41 (21, 22), respectively. Thus, we asked whether both allelic forms of the IGHV1–69 gene segment were equally represented in HIV-1 gp41-reactive Abs elicited by the DNA prime, rAd5 boost vaccine. Of 125 vaccinee-induced IGHV1–69-using Abs, we found that 116 (93%) utilized 54L-HCDR2 variants while only 9 (7%) used 54F-HCDR2 variants (Table 1, Table S5). NGS of pre-vaccination IGHV repertoire demonstrated that all 8 phase Ib and II trial vaccinees encoded 54F and 54L-HCDR2 variants of VH1–69 (Table S12). Therefore, the expression of 54L-HCDR2 VH1–69 variants was not due to vaccinee inability to express 54F-HCDR2 variants. As a comparison, we asked what IGHV1–69 gene allelic variants were used by gp41-reactive Abs in HIV-1 infection. Of 42 IGHV1–69-using gp41-reactive Abs from HIV-1-infected individuals (23), 41/42 (98%) used 54L-HCDR2 variants and 1/42 (2%) used a 54F-HCDR2 variant (Table 1). In contrast, of 64 non-HIV-1-reactive IGHV1–69-using Abs isolated from HIV-1 infected individuals (2, 3, 23), 18/64 (28%) utilized 54L-HCDR2 variants while 46/64 (72%) utilized 54F-HCDR2 variants (Table 1). Finally, of 10 IGHV1–69-using Influenza hemagglutinin Abs isolated from influenza vaccinees or acute influenza infection (24), 9 (90%) utilized 54F-HCDR2 variants and only 1 (10%) utilized a 54L-HCDR2 variant (Table 1). Thus, the DNA prime, rAd5 boost vaccine induced a dominant gp41-reactive Ab response that preferentially used 54L-HCDR2 IGHV1–69 allelic variants, similar to gp41-reactive Abs induced by HIV-1 infection ($p=0.45$; Fisher's Exact Test), but different from influenza-induced IGHV1–69 Abs ($p<0.0001$; Fisher's Exact Test) (Table 1, Table S5).

Since a subset of B-chronic lymphocytic leukemia (B-CLL) Abs uses IGHV1–69 and can cross-react with HIV-1 Env gp41 (23), we compared IGHV1–69 sequences of healthy control Abs (16), B-CLL Abs (23), and gp41-reactive Abs from phase Ib and II trials vaccine-recipients, for CLL archetypes previously described (25). We found that the frequency of CLL archetype matches in our vaccine-induced IGHV1–69-using Abs (1.6%) was no different from healthy control IGHV1–69 sequences (2.3%), but different from CLL Abs (10.6%) (vaccine-induced gp41-reactive vs. CLL Abs, $p=0.002$; vaccine-induced gp41-reactive Abs vs. healthy controls, NS; CLL vs. healthy controls 1.7×10^{-5} , Fisher's Exact test) (Table S13). Thus, there is no evidence of selective derivation of vaccine-induced

gp41-reactive IGWH1–69 antibodies from the same pool of B cells that give rise to CLL Abs.

Functional properties of HIV-1-reactive mAbs

We chose 29 naturally paired heavy and light chain genes for recombinant antibody bulk expression and characterization; 17 gp41-reactive [representing 45 Abs within 17 vaccine-induced clonal lineages (Table S14)] and 12 gp120-reactive [representing 12 clonal lineages (Table S15)] mAbs. Gp41-reactive mAbs bound to rgp140 and/or gp41 proteins via ELISA and/or SPR (Table S16). For Abs that reacted strongly to vaccine Envs, the dissociation constants (K_d) for binding to MN gp41 of 5 gp41 mAbs ranged from ~1 to 71 nM, while binding to vaccine-Env VRC-A and heterologous group M consensus gp140 Envs ranged from ~1 to 23 nM (Table S17). None of the gp41-reactive mAbs mediated neutralization using a panel of pseudotyped HIV-1 isolates: neutralization-resistant (tier 2) VRC-A (A.92RW020) and VRC-C (C.97ZA012), neutralization-sensitive (tier 1) B.HxB2 and B.BaL of VRC-B (B.HxB2/BaL-V3), and heterologous neutralization-sensitive (tier 1) B.MN (Table S18). Only 1/17 (6%) gp41-reactive mAb captured infectious virions (B.NL4-3) (Figure S5A) or mediated ADCC (C.1086) (Figure S6). In contrast, one-third (4/12) of vaccine-induced gp120 mAbs neutralized at least 1 neutralization-sensitive HIV-1 isolate (Table S19); all neutralizing gp120-reactive Abs targeted the third variable loop (V3) (mAbs DH196, DH449, DH450, and DH452) and neutralized HIV-1 B.BaL and/or B.MN. Of the gp120-reactive mAbs, 2/12 (17%) captured infectious HIV-1 (Figure S5B), and 5/12 (42%) mediated ADCC (Figure S7).

Site of reactivity of vaccine-induced gp41 antibodies

We previously reported gp41-reactive Abs from HIV-1 infected patients to be cross-reactive with the 37Kd subunit of bacterial (*E. coli*) RNA polymerase (2). We identified a short region of sequence similarity between gp41 and bacterial RNA polymerase with a shared amino sequence of LRAI (amino-acid (aa) numbering 556–559 in gp41 (PDB: 1AIK) and 43–46 in bacterial RNA polymerase) (PDB: 1BDF) (Figure S8a). A structural alignment of HIV-1 Env gp41 (26) and bacterial RNA polymerase (27) showed that the alpha (α)-subunit helices at the RNA polymerase dimer interface were similar to a portion of the gp41 heptad repeat 1 (HR-1) and heptad repeat 2 (HR-2) helices with a 1.30 Angstroms backbone atom root-mean-square deviation (Figure 8b#x02013;c). This analysis raised the hypothesis that the VRC vaccine-induced gp41-reactive Abs may bind to the gp41 post-fusion structure.

To determine binding sites for vaccine-induced gp41-reactive mAbs on vaccine VRC-A gp140 protein, we proteolytically cleaved the gp140 protein with trypsin, and analyzed the cleavage products on SDS-PAGE gels in western-blot analysis with three VRC-A gp140 and gp41-reactive (DH438, DH440, DH432) and control mAbs, and liquid chromatography-mass spectrometry (LC-MS). We found a ~25kDa fragment of VRC-A gp140 that was blotted by the gp41, VRC-A gp140 and IM-reactive vaccine-induced mAbs and contained a peptide aa sequence (558–567, PDB: 1AIK) AIEAQQHLLQ that placed it in the HR-1 gp41 region overlapping with the LLRAIE gp41 sequence (aa 555–560, PDB: 1AIK) of HR-1 that was a putative cross-reactive region with the bacterial IM-protein, RNA polymerase (Figure

S9). Thus, one region of VRC-A gp140 bound by HIV-1 gp41-reactive mAbs was in the gp41 HR-1 region.

In order to identify additional candidate proteins in IM that cross-reacted with gp41-reactive Abs, SDS-PAGE gels were run with IM-whole cell lysates (WCLs) and analyzed in western blots with VRC-A gp140, gp41 and IM-reactive IGHV1–69 mAbs DH438, DH440, and DH432. Analysis of IM sequences in bands reactive with one or more gp41-reactive mAbs revealed 19 candidate IM proteins by LC-MS (Figure S10a#x02013;b). Alignment of candidate IM protein and gp41 sequences demonstrated one bacterial protein, pyruvate-flavodoxin oxidoreductase, that had sequence similarity to gp41 HR-1 aa 555–560 LLRAIE, with an amino acid sequence in pyruvate-flavodoxin oxidoreductase sequence aa 500–505 LLRGIK (Figure S10c). Thus, pyruvate-flavodoxin oxidoreductase is a second candidate protein that may cross-react with HIV-1 gp41-reactive Abs. That this sequence similarity between gp41 and pyruvate-flavodoxin oxidoreductase was the same as found for the tryptic fragment of DH438-bound VRC-A gp140 (Figure S9), and the sequence similarity found between gp41 and bacterial RNA polymerase (Figures S8) raised the hypothesis that gp41-reactive Abs induced by the VRC vaccine bind to an HR-1 sequence in the gp41 post-fusion conformation. Indeed, vaccine-induced gp41-reactive mAbs DH438, DH432 and DH440 bound to linear peptides containing the LLRAIE HR-1 sequence (Table S20), and 16/17 gp41-reactive mAbs bound a gp41 post-fusion conformation recombinant protein (21) (Table S16).

Polyreactivity of HIV-1-reactive mAbs

Binding to non-HIV-1 molecules was used to identify polyreactivity among these HIV-1-reactive Abs (28, 29). Of the gp41-reactive mAbs, 11/17 (65%) reacted with 1 of 9 non-HIV-1 host proteins and nucleic acids (Table S2, 1, Figure 2a); 4/17 (23%) reacted with mitochondrial inner membrane lipid component, cardiolipin (Table S21), and 5/17 (29%) reacted with HIV-1 uninfected cells (HEp-2 cells) in an immunofluorescence reactivity assay (Table S2, 2, Figure 2b). We found DNA prime, rAd5-boost vaccine-induced gp41-reactive Abs to be reactive with WCLs of anaerobic (14/17, 82%) or aerobic (13/17, 76%) IM. Forty-seven percent of gp41-reactive Abs were reactive with the 37Kd subunit of *E. coli* RNA polymerase that has been shown to cross-react with HIV-1 gp41-reactive Abs (2) (Figure S11a, S11c; Figure 2c). In contrast, of the gp120-reactive mAbs, 4/12 (33%) were reactive with 1 of 9 host proteins and nucleic acids (Table S21), 1/12 (8%) with cardiolipin (Table S21), 1/12 (8%) with HEp-2 cells (Table S22), 8/12 (67%) with anaerobic IM-WCL, 5/12 (42%) with aerobic IM-WCL, and 2/12 (17%) with *E. Coli* RNA polymerase (Figure S11b, S11c). Collectively, DNA prime, rAd5 boost vaccine-induced gp41-reactive mAbs were more polyreactive than gp120-reactive mAbs ($p < 0.003$, Cochran-Mantel-Haenszel Test) (Table 2).

NGS of pre-vaccine IgHV repertoires

The isolation of Abs from HIV-1-infected and naïve individuals that cross-reacted with gp41 and IM-WCLs led to the hypothesis that a pool of B cells exist that are cross-reactive with Env gp41 and IM (2, 3). To determine if these types of naïve B cells can respond in the

setting of HIV-1 Env gp140 vaccination, we performed immunoglobulin variable heavy chain NGS on pre-vaccination blood mononuclear cell-derived RNA samples from the 8 vaccinees from which post-vaccine Env-specific memory B cells were isolated. The immunoglobulin repertoire of IGHV1-IGHV6 gene families for IgA, G, and M isotypes were probed in pre-vaccination blood B cells (Table S24). We used the V_HDJ_H DNA sequences of all post-vaccination Abs with unique V_HDJ_H rearrangements from each of the 8 vaccinees to search their pre-vaccination B-cell repertoire for V_HDJ_H rearrangements that belonged to members of the same clonal lineages. We tested the relatedness among sequences by fitting alternative phylogenetic models in which the sequences were or were not hypothesized to share a common ancestor. In both models, we computed a Bayesian average over ancestral sequences; two such ancestors in the model for similar sequences derived from different B cells, one ancestor for the model for clonally-related sequences. In the latter case, we also averaged over all unobserved intermediates as well. The model with the larger summed likelihood was selected.

From vaccinee 082-003, we found a pre-vaccination IgM V_HDJ_H rearrangement (DH477) that was clonally related to a post-vaccination gp41-reactive IgG1 Ab, DH476. Direct comparison of these sequences revealed shared junctional sequences, identical HCDR3 length and 85% HCDR3 nucleotide sequence homology (Figure 3a#x02013;b, Figure S12), demonstrating that pre-vaccination DH477 and post-vaccination DH476 Abs came from the same B cell precursor. In addition to being reactive with all three recombinant vaccine-Envs, the post-vaccination Ab DH476 was reactive with rgp41 and with both anaerobe and aerobic IM-WCLs (Figure 3c–d, f). The pre-vaccine clonal lineage member DH477 was an unmutated IgM Ab complemented by the post-vaccine DH476 light chain. Pre-vaccine Ab DH477 reacted with vaccine strain Env, MN gp41, and host and/or environmental antigens including IM (Figure 3c–f; Table S25–26). The pre-vaccine Ab DH477 had greater reactivity to IM than the post-vaccine Ab DH476 (Figure 3f), and only bound VRC-A Env surface-expressed on 293 cells (Figure 3e), but did not bind VRC-A, B or C gp140 recombinant proteins (Figure 3c). Reactivity with IM-WCLs decreased from pre-vaccination Ab DH477 to post-vaccination Ab DH476, whereas increased binding of vaccine Env gp140 and post-fusion forms of gp41 was associated with vaccine Env-induced affinity maturation (Figure 3c#x02013;f). Thus, vaccine-Env gp41-reactive Ab response can indeed arise from a pool of IM-gp41 cross-reactive B cells that undergo post-vaccination affinity maturation.

Discussion

In this study we found that the DNA prime, rAd5 boost HIV-1 vaccine induced a non-neutralizing dominant Env gp41-reactive Ab response derived from a polyreactive B cell pool. These findings have important implications for HIV-1 vaccine design. The isolation of gp41 and IM-reactive B cells from uninfected individuals (2, 3) raised the hypothesis that the HIV-1 gp41-reactive Ab response in HIV-1 infection may in part result from HIV-1 gp41 stimulating a pre-infection pool of B cells cross-reactive with IM and Env. We confirmed this hypothesis by the identification of a pre-vaccination IM, VRC-A gp140, and gp41-reactive Ab that was clonally related to a vaccine-induced Env-reactive Ab (Figure 3).

The pre-vaccination gp41-reactive Ab DH477 was an unmutated IgM Ab, suggesting it represented the receptor of a naïve B cell. While the great majority of human IgM⁺, IgD⁺ B cells are naïve B cells that are unmutated, a minority of IgM⁺, IgD⁺ memory B cells are unmutated as well (30, 31). Thus, DH477 could have been derived from an IgM⁺, IgD⁺ naïve B cell, but we cannot rule out as well that DH477 was derived from an unmutated IgM⁺, IgD⁺ memory B cell. That the pre- (DH477) and post- (DH476) vaccine Abs reacted with IM-WCLs, gp41 and vaccine-Env proteins, suggested that IM or other environmental antigens may stimulate gp41-reactive B cells prior to vaccination that can react with the vaccine Env, but does not prove that all such Abs arose in this manner. B cell development in mice has been demonstrated to occur in the intestinal mucosa, and is regulated by extracellular signals from IM that influence the gut immunoglobulin repertoire (32). Our findings also implicate IM imprinting of B cell development in humans that can impact the quality of an HIV vaccine Ab response.

Here we demonstrated that a HIV-1 Env-gp140 vaccine containing both gp120 and gp41 primarily induced a gp41-dominant Ab response, whereas previous gp120-only vaccines in humans (14) and rhesus macaques (15) induced a robust gp120-reactive memory B cell response. It has been demonstrated that B cells with higher affinity for an antigen can outcompete other B cells with lower affinity for the same antigen (33–35), and B cells *in vitro* with similar, but slightly different B cell receptors can compete for antigen binding (36). Thus, our data raise the additional hypothesis that the dominant non-neutralizing IM cross-reactive gp41-reactive Abs out-competed gp120-reactive Abs.

Of interest, the gp41-reactive Ab response was predominantly restricted to IGHV1–69 gene segment usage. Previously, IGHV1–69 has been noted for influenza stalk (20, 37), HIV-1 gp41 hydrophobic pocket (21), and membrane proximal region Ab responses (38). That the gp41 Ab response constituted predominantly IGHV1–69 memory B cells that utilized the 54L-HCDR2 IGHV1–69 alleles, suggested that the 54L-HCDR2 IGHV1–69 gene allele variant was the predominant IGHV better able to structurally recognize the dominant gp41 epitope expressed by the DNA prime, rAd5 boost vaccine. However, it is important to note that pre-vaccine Ab DH477 used 54F-HCDR2 variant of VH1–69 (Figure 3, Figure S12), while the clonally-related post-vaccine Ab DH476 used 54L VH1–69 variant (Table S5, Figure S12). Thus, a second mechanism of 54L VH1–69-usage was via somatic hypermutations to recognize gp41 epitopes presented by gp140 immunogens in the DNA prime, rAd5 boost vaccine.

One hypothesis for vaccine optimization is to utilize a native trimer containing gp41 to attempt to induce broadly-reactive neutralizing antibodies (bnAbs) (39, 40). These immunogens contain the recently described gp41-gp120 conformational bnAbs epitopes (41–44), but not the membrane proximal portion of gp41 neutralizing epitopes (45, 46). The Env insert in the VRC Env DNA-Ad5 vaccine had a partial deletion in the C-C loop of gp41, unlike the native Env. While it has been reported that another HIV-1 Env vaccine with the gp41 C-C loop intact has also induced high levels of gp41-reactive Abs (47), this vaccine has not been studied in an efficacy trial nor have its vaccine-induced gp41-reactive mAbs been characterized, including for environmental or IM cross-reactivity. In non-human primate studies, gp41-reactive plasma Abs have been reported to be both protective (48, 49)

and non-protective (50, 51). Defining the B cell compartment from which polyreactive gp41-reactive B cells originate, and determining how they are regulated are important areas of future study.

Thus, as in HIV-1 infection, Env vaccination can induce polyreactive, non-neutralizing gp41-reactive Ab repertoire responses from pre-existing B cells that can be cross-reactive with IM. Since the B cell repertoire can be imprinted at birth by IM antigens (32), our data raise the hypothesis that neonatal immunization with HIV-1 Env may be able to imprint the B cell repertoire to respond to Env antigenic sites that may otherwise be subdominant or disfavored, including Env broadly neutralizing Ab epitopes (52, 53). In this regard, Goo et al. recently reported that HIV-1 infected infants can make broadly neutralizing Abs, and in some cases such Abs arise within the first year of life (54).

Finally, if similar diversion towards dominant polyreactive gp41-reactive Abs is found with other gp41-containing vaccine regimens with intact gp41, the data in this study suggest that one region of gp41 in vaccine immunogens that might be considered for deletion or modification to avoid IM-gp41 cross-reactivity in the setting of vaccination includes amino acids in the gp41 HR-1 region.

Materials and Methods

Clinical trial samples

Vaccine-induced antibody (Ab) repertoires were studied four weeks post final vaccination (rAd5 boost) in plasma and blood derived memory B cells of 4 HVTN 204 (5), and 4 HVTN 082 (Protocol HVTN 082; DIADS Document ID 10771) trial participants, and in plasma only from an additional 40 HVTN 505 vaccine recipients (11). Vaccinees were HIV-1 seronegative adults enrolled in the phase Ib HVTN 082 (Protocol HVTN 082; DIADS Document ID 10771) and phase II HVTN 204 (Protocol HVTN 204; BB IND 12326 HELD BY DAIDS) (5) trials that studied the DNA prime, rAd5 boost vaccine. From n=480 (HVTN 204), the 4 participants chosen for our study displayed plasma binding reactivity with vaccine strain Envs, and/or neutralization of clade C MW965 HIV-1 isolate. From n=8 (HVTN 082, 4 twin pairs), we selected one individual from each twin pair to study genetically different subjects. All study samples were obtained by informed consent and all studies were approved by the Duke University Institutional Review Board.

Flow cytometry memory B cell single cell sorting

Single-cell isolation of memory B cells decorated with both AlexaFluor® 647 and Brilliant Violet 421-tagged HIV-1 VRC-A (A.92RW020), VRC-B (B.HxB2/BaL-V3), VRC-C (C.97ZA012), or CON-S Env gp140s or gp120s was performed using a BD FACSAria™ or a BD FACSAria™ II (BD Biosciences, San Jose, CA), and the flow cytometry data were analyzed using FlowJo (Treestar, Ashland, OR) (13, 14, 55).

Ab binding and epitope mapping

Recombinant monoclonal antibodies (mAbs) were screened for binding specificities to multiclade HIV-1 gp120 Envs and clade B.MN gp41 (Product #10911, ImmunoDX,

Woburn, MA) via standard enzyme-linked immunosorbent assays (ELISA) (56). Gp120-reactive Abs bound both Env gp140 and gp120 recombinant proteins, but gp41-reactive Abs bound recombinant MN gp41 protein or differentially to CON-S and/or VRC A Envs (CON-S gp140+, CON-S gp120-, VRC-A gp140+, VRC-A gp120). MAb were epitope-mapped on multiclade consensus and primary isolates gp140 overlapping peptide sets, including HIV-1 MN and B.CON gp140 obtained from the NIH AIDS Reagent Repository, via ELISA and linear peptide array (57). Binding antibody multiplex assay (BAMA) standardized custom binding Ab multiplex assay was used to determine reactivity of serum and recombinant mAbs with antigens (1). Pre-vaccinated serum samples and anti-RSV mAb (palivizumab) were used to establish plasma and mAb binding cutoffs, respectively, in BAMA. For SPR analysis, Env gp140 (A.92RW020, B.HxB2/Bal, C.97ZA012, M.CON-S), 5-Helix gp41 (21) and MN gp41 proteins (Product #10911, ImmunoDX, Woburn, MA) binding was measured by injecting Env proteins at varying concentration (0.5–500 µg/mL) over each mAb captured on anti-human IgFc immobilized antibody (Millipore) on a CM5 sensor surface as previously described (58). Non-specific binding to the control palivizumab mAb and signal drifts from phosphate buffered saline (PBS) were used for double referencing and to measure specific binding responses. Rate constants (k_a , k_d) were measured by global curve fitting to a 1:1 Langmuir model as described previously (58). For gp41 proteins (MN gp41 and 5-helix) binding to pre-vaccine Abs, rate constants could not be reliably measured due to saturation of binding responses at a relatively lower antigen concentrations and K_d values were measured using steady-state analysis. Post vaccine Abs did not show this limitation when binding to either MN gp41 or 5-helix gp41 proteins (Figure 3).

Ab staining of cell-surface expressed HIV-1 VRC-A gp145

For the surface expression of VRC-A gp145, 293i cells were transfected with pHV130770 plasmids using ExpiFectamine™ 293 Transfection Kits (Life Technologies Cat. # A14525) following the manufactures' protocol. The cells were washed with four volumes of room-temperature phosphate-buffered saline (DPBS) (Life Technologies Cat # 10010-023), and centrifuged (500g for 5 minutes). 10^6 cells were resuspended in 80 µL of staining buffer (1% BSA in DPBS) to which were added 20 µL of diluted antibodies (1 µg per reaction). After incubation (1 hour at 4°C) with shaking, cells were washed and centrifuged (500 X g). Cells were incubated with either 20 µL isotype-APC control (BD cat #555751) or 20 µL anti-Human IgG-APC (BD cat #550931) for 30–45 minutes at 4°C, protected from light. After washing and fixation, cells were analyzed by flow cytometer (BD LSRFortessa).

Ab polyreactivity

Vaccine-induced polyreactivity of Abs was assessed using commercially available kits (AtheNA Multi-Lyte® System and HEp-2 cell immunofluorescence assay, Zeus Scientific; anti-cardiolipin ELISA - QUANTA Lite® ACA IgG III, Inova Diagnostics, San Diego, CA) (3). Reactivity with whole cell lysates (WCL) of anaerobic and aerobic intestinal microbiota (IM) extracts of stool specimens (3) were determined via BAMA and western blot (WB) as described previously (2). For WB analysis, 20µg/mL of mAbs was used to test reactivity with 100µg of IM WCLs. SPR analysis of IM WCL samples were performed on a BIAcore 3000 instrument (GE Healthcare) as previously described (2), with some modifications. IM

WCL samples were spun down to remove aggregates and each lysate samples (total protein concentrations of 7.87 and 7.97 mg/mL for aerobic and anaerobic lysates respectively) were diluted in PBS buffer (1:1 or 1:2) and injected over each mAb captured over an anti-human IgFc Ab immobilized on a C1 sensor chip. Specific binding of the lysate proteins to the mAbs were measured after subtraction of non-specific binding to anti-HA mAb CH65 and binding responses were measured at post-injection report point (10s following end of injection).

Proteomic analysis of IM proteins and HIV-1 VRC-A gp140

SDS-PAGE gels were run with IM and western blot analysis performed with 3 VRC-A gp140 and MN gp41-reactive, IM cross-reactive VH1-69-using mAbs; DH440, DH432 and DH438. Bands were cut from gels that were reactive with one or more of these mAbs in western blot, and performed proteomic analysis by liquid chromatography-mass spectrometry (LC-MS) of tryptic fragments of these protein bands to identify the reactive protein. One of these bands (An1) was recognized by all 3 gp41-reactive mAbs and an identified protein in the WB reactive band An1 (Figure S10) was bacterial pyruvate-flavodoxin oxidoreductase (NCBI accession #: WP_022497386).

In order to directly determine where on the VRC-A gp140 protein the vaccine-induced gp41 mAbs bound, we proteolytically cleaved the gp140 protein with trypsin, and then analyzed by WB analysis the cleavage products. One of the reactive bands (~25kDa) that blotted with the gp41-reactive mAb tested was identified by LC-MS proteomic analysis following reduction and alkylation.

Sequence and structural similarities of HIV-1 Env gp41 and bacterial proteins

Alignment of bacterial protein identified by LC-MS with gp41 Env sequences was assessed by BLAST analysis of the VRC-A, VRC-B and VRC-C gp140 and MN gp41 amino acid sequences against sequences of 19 candidate proteins identified in IM by LC-MS. We used a cutoff E-value of <1 to allow for short stretches of sequence matches. The BLAST search returned only one similar region in pyruvate flavodoxin oxidoreductase where sequence similarity was observed below the E-value cutoff between bacterial proteins and the gp41 ectodomain. The H1 helices at the homodimer interface of the alpha subunit of RNA polymerase (PDB: 1BDF) were structurally aligned to the 6-helix bundle conformation of gp41 (PDB: 1AIK) using an implementation of the Kabsch algorithm.

Ab functional characterization

Purified mAbs were screened for neutralization via TZM-bl assay (59, 60), Ab dependent cellular cytotoxicity (ADCC) (61, 62), and infectious virion capture (63), of HIV-1 isolates.

PCR isolation of heavy and light chain genes

Heavy (IGHV) and light (IGKV, IGLV) chain genes were isolated via single cell PCR (13, 64) and the sequences computationally determined as described (65–67). Inferences of the genetic features of the immunoglobulin IGHV, IGKV, and IGLV sequences were previously described (68, 69).

Expression of IGHV, IGKV, and IGLV chains as full-length IgG1 rmAbs

Plasmids encoding the IGHV, IGKV, and IGLV genes were generated and used for rmAb production in human embryonic kidney cell lines (ATCC, Manassas, VA) (13, 38), via small-scale transfection and as purified mAbs in larger quantities (70, 71). Purified rmAbs were dialyzed against PBS, analyzed, and stored at 4°C.

Clonal lineage determination

Sequences were subject to statistical analysis for lineage membership. Briefly, sequences were organized into *clans*, by definition sharing inferred IGHV and IGHJ genes and CDRH3 length. Within these clans, we used agglomerative clustering using a Bayesian phylogenetic merit function as follows. To calculate the score for the hypothesis that a given sequence is a member of a lineage L, we compute the posterior likelihoods under the phylogenetic hypotheses that they are and are not members of the same clone. The Bayes factor for this comparison is the objective function for clustering. Sequences were taken in succession, and placed into the lineage (including an empty lineage, i.e., founding a new lineage) that maximizes the objective function. The automated inference was followed up by visual inspection of the DNA sequence alignments for confirmation.

Statistical analysis

Statistically significant differences in the profiles of gp120-reactive and gp41-reactive Abs were determined via various tests (as indicated for each table and figure) in SAS (SAS v9.3). A p-value <0.05 determined statistical significance.

Next generation sequencing (NGS)

PBMC-extracted RNA was used to generate cDNA amplicons for pyrosequencing (Illumina). RNA isolated from vaccinee PBMCs was separated into two equal aliquots prior to cDNA production; cDNA amplification and NGS were performed on both aliquots as independent samples. IGHV genes were amplified via a modification of the processes previously described (13). The RT reaction was carried out in 30 µL reaction mixtures at 55 °C for 1 h after addition of 50 units/reaction Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA), 40 units/reaction RNaseOUT (Invitrogen, Carlsbad, CA), 25 mM dNTPs (Invitrogen, Carlsbad, CA), 5x first strand buffer (Invitrogen, Carlsbad, CA), 0.1 mM DTT (Invitrogen, Carlsbad, CA), and 25 µM human IgA, IgG, and IgM constant region primers as previously described (13). After cDNA synthesis, IGHV1-IGHV6 genes were amplified separately for IgA, IgG and IgM isotypes by two rounds of PCR in 96-well PCR plates in 50 µL reaction mixtures. The first-round of PCR contained 5 µL of RT reaction products, 1 unit of iProof DNA polymerase (Biorad), 10 µL 5X iProof GC Buffer(Biorad), 10 mM dNTPs (Invitrogen, Carlsbad, CA), 50 mM MgCl₂ (Biorad) and 25 µM of either IgA, IgG and IgM constant region primers and sets of IGHV-IGHV6 variable region primers (Table S23). The first round of PCR was performed at 98°C × 1 min followed by 25 cycles of 98°C × 15 s, 60°C × 15 s, 72°C × 35 s, and one cycle at 72°C × 7min. First round PCR products were purified using QIAquick PCR purification kit (Qiagen) and eluted into 50 µL DNase/RNase free distilled water. Nested second round PCR was performed in 50ul reaction mixture with 5 µL of purified first-round PCR product, 1.25Units/reaction Platinum Taq DNA

Polymerase High Fidelity (Invitrogen, Carlsbad, CA), 10mM dNTPs, and 5ul of Nextera index kit barcode-tagged primers (Illumina). During the second round of nested PCR, the IGHV1-IGHV6 primers were amplified in separate reaction mixes for each variable region primer. The second-round of PCR was performed at 94°C × 2min followed by 3 cycles of 94°C × 15 s, 55°C × 30 s, 68°C × 30 s, 7 cycles of 94°C × 15 s, 60°C × 30 s, 68°C × 30 s, and one cycle at 68°C × 10 mins. Samples of IGHV chain PCR products were analyzed on 2% agarose gels prior to total sample purification via gel extraction (QIAquick gel extraction kit; Qiagen) and eluted into 25 µL DNase/RNase free distilled water. 5 µL of IGHV1-IGHV6 cDNA amplicons were pooled for each Ig isotype. Pooled IgA, IgG and IgM cDNA amplicons were quantified using the KAPA SYBR FAST qPCR kit (KAPA Biosystems). 4 nM of IgA, IgG and IgM samples was selected for NGS. IgA, IgG and IgM samples were denatured with NaOH (1x) and then mixed with hybridization buffer. Denatured samples were mixed with 35% (by volume) PhiX control/non-relevant DNA (Illumina) in a final reaction mixture, from which 600 µL was loaded to Illumina Miseq kit v3 – 600 cycles PE for amplification. We have previously shown that the primer set used here does not induce primer IGHV bias (3).

NGS-generated sequences were processed and analyzed computationally according to the following protocol; Illumina reads were discarded if they did not meet the following criteria:

30 Phred quality score (corresponding to 99.9% base call accuracy) for at least 95% of all base positions in the sequence. Primer sequences were removed from the reads and only unique sequences were retained. The unique sequences were then analyzed using the Cloanlyst software suite (72) to infer the IGHV rearrangements (V_HDJ_H) and determine clonal relatedness. Illumina next generation sequencing was performed independently on duplicate samples (RNA samples split into 2 aliquots for independent cDNA production, amplification, and deep sequencing). Illumina runs were performed using independent Illumina kits. For the purpose of gene counting, only sequences that appeared in both duplicate runs (no mismatches in the coding region) were used (Tables S12, S24). For the identification of pre-vaccine lineage members, replication was not required. The statistical methods we used are not sensitive to random sequencing error and the likelihood that sequencing errors would produce artifactual apparent lineage membership in an otherwise unrelated sequences is negligible.

We used NGS to study the pre-vaccination samples of all 8 vaccinees in the study, and we found three vaccinees whose pre-vaccination samples had antibodies that were in the same antibody clan (same V_HDJ_H and HCDR3 length and similarity), but two of these pre-vaccination antibodies had ambiguities in their junctional sequences and shared mutations vs. allelic differences, such that a definitive conclusion could not be reached that the pre-vaccination and post-vaccination V_HDJ_H originated from the same B cell. However, in the case of the pre-vaccination- DH477 and post-vaccination- DH476 antibodies, from the shared mutations and junctional sequences we could definitively state that they arose from the same B cell. In order to investigate the binding specificities of the pre- (DH477) and post- (DH476) vaccine antibodies, pre- and post-vaccine V_H chains were paired with the natural light chain of the post-vaccine antibody.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Garnett Kelsoe for manuscript review, Lawrence Armand for production of fluorophore- labeled reagents, and Christina Stolarchuk, Shelley Stewart, Amy Wang, Ryan Duffy, Aaron Deal, Joshua Eudailey, Tarra Von Holle, James Alin, Letealia Oliver, Frederick Jaeger, and Sabrina Arora for technical assistance. The data presented in this manuscript are tabulated in the main paper and in the supplementary materials. Sequences for the antibodies reported in this study are deposited in GenBank accession numbers X-Y. Research materials used in this study are available from Duke University upon request and subsequent execution of an appropriate materials transfer agreement. Supported by NIH, NIAID UM-1 grant Center For HIV/AIDS Vaccine Immunology- Immunogen Discovery (CHAVI-ID; UM1 AI100645), NIH NIAID Duke University Center for AIDS Research (CFAR; P30-AI-64518), the NIH NIAID HVTN Laboratory Center UM1AI068618, and the intramural research program of the Vaccine Research Center, National Institute of Allergy and Infectious Diseases, NIH. WBW designed and performed experiments, analyzed data and co-wrote the paper; HXL, KJ, MAM, DMJ, JFW performed isolation of antibodies, reviewed data and edited the paper; TBK, AH, KW, KS, AMT performed computational analysis of antibody sequences; FG, RZ, HS sequenced antibodies and performed NGS; SMA, PL, MZT, KS, XS, AF, KEL, RP performed antibody binding and functional assays; JP, GF performed ADCC assays; NV, DG, PG performed statistical analysis; DCM performed neutralization assays; MES, SH, SK, NG, JM, JRM, RK, LC, GBN, CM, GC, JM, MK, BSG, LRB were members of the VRC or HVTN teams that carried out the clinical trials; GDT analyzed data, designed experiments, reviewed data and edited the paper; BFH conceived and designed the study, performed ANA analysis, reviewed all data, wrote the paper.

References and Notes

1. Tomaras GD, et al. Initial B-cell responses to transmitted human immunodeficiency virus type 1: virion-binding immunoglobulin M (IgM) and IgG antibodies followed by plasma anti-gp41 antibodies with ineffective control of initial viremia. *J Virol.* 2008 Dec.82:12449. [PubMed: 18842730]
2. Trama AM, et al. HIV-1 Envelope gp41 Antibodies Can Originate from Terminal Ileum B Cells that Share Cross-Reactivity with Commensal Bacteria. *Cell Host Microbe.* 2014 Aug.16:215. [PubMed: 25121750]
3. Liao HX, et al. Initial antibodies binding to HIV-1 gp41 in acutely infected subjects are polyreactive and highly mutated. *J Exp Med.* 2011 Oct.208:2237. [PubMed: 21987658]
4. Catanzaro AT, et al. Phase I clinical evaluation of a six-plasmid multiclade HIV-1 DNA candidate vaccine. *Vaccine.* 2007 May.25:4085. [PubMed: 17391815]
5. Churchyard GJ, et al. A phase IIA randomized clinical trial of a multiclade HIV-1 DNA prime followed by a multiclade rAd5 HIV-1 vaccine boost in healthy adults (HVTN204). *PLoS One.* 2011; 6:e21225. [PubMed: 21857901]
6. Kibuuka H, et al. A phase 1/2 study of a multiclade HIV-1 DNA plasmid prime and recombinant adenovirus serotype 5 boost vaccine in HIV-Uninfected East Africans (RV 172). *J Infect Dis.* 2010 Feb.201:600. [PubMed: 20078213]
7. Jaoko W, et al. Safety and immunogenicity study of Multiclade HIV-1 adenoviral vector vaccine alone or as boost following a multiclade HIV-1 DNA vaccine in Africa. *PLoS One.* 2010; 5:e12873. [PubMed: 20877623]
8. Graham BS, et al. Phase 1 safety and immunogenicity evaluation of a multiclade HIV-1 DNA candidate vaccine. *J Infect Dis.* 2006 Dec.194:1650. [PubMed: 17109336]
9. Catanzaro AT, et al. Phase 1 safety and immunogenicity evaluation of a multiclade HIV-1 candidate vaccine delivered by a replication-defective recombinant adenovirus vector. *J Infect Dis.* 2006 Dec. 194:1638. [PubMed: 17109335]
10. Koup RA, et al. Priming immunization with DNA augments immunogenicity of recombinant adenoviral vectors for both HIV-1 specific antibody and T-cell responses. *PLoS One.* 2010; 5:e9015. [PubMed: 20126394]
11. Hammer SM, et al. Efficacy trial of a DNA/rAd5 HIV-1 preventive vaccine. *N Engl J Med.* 2013 Nov.369:2083. [PubMed: 24099601]

12. Liao HX, et al. A group M consensus envelope glycoprotein induces antibodies that neutralize subsets of subtype B and C HIV-1 primary viruses. *Virology*. 2006 Sep.353:268. [PubMed: 17039602]
13. Liao HX, et al. High-throughput isolation of immunoglobulin genes from single human B cells and expression as monoclonal antibodies. *J Virol Methods*. 2009 Jun.158:171. [PubMed: 19428587]
14. Moody MA, et al. HIV-1 gp120 vaccine induces affinity maturation in both new and persistent antibody clonal lineages. *J Virol*. 2012 Jul.86:7496. [PubMed: 22553329]
15. Wiehe K, et al. Antibody light-chain-restricted recognition of the site of immune pressure in the RV144 HIV-1 vaccine trial is phylogenetically conserved. *Immunity*. 2014 Dec.41:909. [PubMed: 25526306]
16. Boyd SD, et al. Individual variation in the germline Ig gene repertoire inferred from variable region gene rearrangements. *J Immunol*. 2010 Jun 15.184:6986. [PubMed: 20495067]
17. Sasso EH, Johnson T, Kipps TJ. Expression of the immunoglobulin VH gene 51p1 is proportional to its germline gene copy number. *J Clin Invest*. 1996 May.97:2074. [PubMed: 8621797]
18. Abecasis GR, et al. A map of human genome variation from population-scale sequencing. *Nature*. 2010 Oct.467:1061. [PubMed: 20981092]
19. Lerner RA. Rare antibodies from combinatorial libraries suggests an S.O.S. component of the human immunological repertoire. *Mol Biosyst*. 2011 Apr.7:1004. [PubMed: 21298133]
20. Xu R, et al. A recurring motif for antibody recognition of the receptor-binding site of influenza hemagglutinin. *Nat Struct Mol Biol*. 2013 Mar.20:363. [PubMed: 23396351]
21. Luftig MA, et al. Structural basis for HIV-1 neutralization by a gp41 fusion intermediate-directed antibody. *Nat Struct Mol Biol*. 2006 Aug.13:740. [PubMed: 16862157]
22. Sabin C, et al. Crystal structure and size-dependent neutralization properties of HK20, a human monoclonal antibody binding to the highly conserved heptad repeat 1 of gp41. *PLoS Pathog*. 2010; 6:e1001195. [PubMed: 21124990]
23. Hwang KK, et al. IGHV1-69 B cell chronic lymphocytic leukemia antibodies cross-react with HIV-1 and hepatitis C virus antigens as well as intestinal commensal bacteria. *PLoS One*. 2014; 9:e90725. [PubMed: 24614505]
24. Moody MA, et al. H3N2 influenza infection elicits more cross-reactive and less clonally expanded anti-hemagglutinin antibodies than influenza vaccination. *PLoS One*. 2011; 6:e25797. [PubMed: 22039424]
25. Darzentas N, et al. A different ontogenesis for chronic lymphocytic leukemia cases carrying stereotyped antigen receptors: molecular and computational evidence. *Leukemia*. 2010 Jan.24:125. [PubMed: 19759557]
26. Chan DC, Fass D, Berger JM, Kim PS. Core structure of gp41 from the HIV envelope glycoprotein. *Cell*. 1997 Apr.89:263. [PubMed: 9108481]
27. Zhang G, Darst SA. Structure of the Escherichia coli RNA polymerase alpha subunit amino-terminal domain. *Science*. 1998 Jul.281:262. [PubMed: 9657722]
28. Mouquet H, Nussenzweig MC. Polyreactive antibodies in adaptive immune responses to viruses. *Cell Mol Life Sci*. 2012 May.69:1435. [PubMed: 22045557]
29. Haynes BF, et al. Cardiolipin polyspecific autoreactivity in two broadly neutralizing HIV-1 antibodies. *Science*. 2005 Jun.308:1906. [PubMed: 15860590]
30. Klein U, Küppers R, Rajewsky K. Evidence for a large compartment of IgM-expressing memory B cells in humans. *Blood*. 1997 Feb.89:1288. [PubMed: 9028952]
31. Klein U, Küppers R, Rajewsky K. Human IgM+IgD+B cells, the major B cell subset in the peripheral blood, express V kappa genes with no or little somatic mutation throughout life. *Eur J Immunol*. 1993 Dec.23:3272. [PubMed: 8258343]
32. Wesemann DR, et al. Microbial colonization influences early B-lineage development in the gut lamina propria. *Nature*. 2013 Sep.501:112. [PubMed: 23965619]
33. Dal Porto JM, Haberman AM, Kelsoe G, Shlomchik MJ. Very low affinity B cells form germinal centers, become memory B cells, and participate in secondary immune responses when higher affinity competition is reduced. *J Exp Med*. 2002 May.195:1215. [PubMed: 11994427]

34. Shih TA, Meffre E, Roederer M, Nussenzweig MC. Role of BCR affinity in T cell dependent antibody responses in vivo. *Nat Immunol.* 2002 Jun.3:570. [PubMed: 12021782]
35. Dal Porto JM, Haberman AM, Shlomchik MJ, Kelsoe G. Antigen drives very low affinity B cells to become plasmacytes and enter germinal centers. *J Immunol.* 1998 Nov.161:5373. [PubMed: 9820511]
36. McGuire AT, et al. HIV antibodies. Antigen modification regulates competition of broad and narrow neutralizing HIV antibodies. *Science.* 2014 Dec.346:1380. [PubMed: 25504724]
37. Sui J, et al. Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. *Nat Struct Mol Biol.* 2009 Mar.16:265. [PubMed: 19234466]
38. Morris L, et al. Isolation of a human anti-HIV gp41 membrane proximal region neutralizing antibody by antigen-specific single B cell sorting. *PLoS One.* 2011; 6:e23532. [PubMed: 21980336]
39. Julien JP, et al. Crystal structure of a soluble cleaved HIV-1 envelope trimer. *Science.* 2013 Dec. 342:1477. [PubMed: 24179159]
40. Lyumkis D, et al. Cryo-EM structure of a fully glycosylated soluble cleaved HIV-1 envelope trimer. *Science.* 2013 Dec.342:1484. [PubMed: 24179160]
41. Falkowska E, et al. Broadly neutralizing HIV antibodies define a glycan-dependent epitope on the prefusion conformation of gp41 on cleaved envelope trimers. *Immunity.* 2014 May.40:657. [PubMed: 24768347]
42. Blattner C, et al. Structural delineation of a quaternary, cleavage-dependent epitope at the gp41-gp120 interface on intact HIV-1 Env trimers. *Immunity.* 2014 May.40:669. [PubMed: 24768348]
43. Scharf L, et al. Antibody 8ANC195 reveals a site of broad vulnerability on the HIV-1 envelope spike. *Cell Rep.* 2014 May.7:785. [PubMed: 24767986]
44. Huang J, et al. Broad and potent HIV-1 neutralization by a human antibody that binds the gp41-gp120 interface. *Nature.* 2014 Sep.
45. Muster T, et al. Cross-neutralizing activity against divergent human immunodeficiency virus type 1 isolates induced by the gp41 sequence ELDKWAS. *J Virol.* 1994 Jun.68:4031. [PubMed: 7514684]
46. Huang J, et al. Broad and potent neutralization of HIV-1 by a gp41-specific human antibody. *Nature.* 2012 Nov.491:406. [PubMed: 23151583]
47. Goepfert PA, et al. Specificity and 6-month durability of immune responses induced by DNA and recombinant modified vaccinia Ankara vaccines expressing HIV-1 virus-like particles. *J Infect Dis.* 2014 Jul.210:99. [PubMed: 24403557]
48. Li Q, et al. Live simian immunodeficiency virus vaccine correlate of protection: local antibody production and concentration on the path of virus entry. *J Immunol.* 2014 Sep.193:3113. [PubMed: 25135832]
49. Robinson HL. Non-neutralizing antibodies in prevention of HIV infection. *Expert Opin Biol Ther.* 2013 Feb.13:197. [PubMed: 23130709]
50. Burton DR, et al. Limited or no protection by weakly or nonneutralizing antibodies against vaginal SHIV challenge of macaques compared with a strongly neutralizing antibody. *Proc Natl Acad Sci U S A.* 2011 Jul.108:11181. [PubMed: 21690411]
51. Kwa S, et al. CD40L-Adjuvanted DNA/MVA SIV Vaccine Enhances Protection Against Neutralization Resistant Mucosal SIV Infection. *J Virol.* 2015 Feb.
52. Haynes BF, Verkoczy L. AIDS/HIV. Host controls of HIV neutralizing antibodies. *Science.* 2014 May.344:588. [PubMed: 24812389]
53. Tomaras GD, Haynes BF. Lessons from babies: inducing HIV-1 broadly neutralizing antibodies. *Nat Med.* 2014 Jun.20:583. [PubMed: 24901564]
54. Goo L, Chohan V, Nduati R, Overbaugh J. Early development of broadly neutralizing antibodies in HIV-1-infected infants. *Nat Med.* 2014 Jun.20:655. [PubMed: 24859529]
55. Gray ES, et al. Isolation of a monoclonal antibody that targets the alpha-2 helix of gp120 and represents the initial autologous neutralizing-antibody response in an HIV-1 subtype C-infected individual. *J Virol.* 2011 Aug.85:7719. [PubMed: 21613396]

56. Liao HX, et al. Vaccine induction of antibodies against a structurally heterogeneous site of immune pressure within HIV-1 envelope protein variable regions 1 and 2. *Immunity*. 2013 Jan.38:176. [PubMed: 23313589]
57. Friedman J, et al. Isolation of HIV-1-neutralizing mucosal monoclonal antibodies from human colostrum. *PLoS One*. 2012; 7:e37648. [PubMed: 22624058]
58. Alam SM, et al. Antigenicity and immunogenicity of RV144 vaccine AIDSVAX clade E envelope immunogen is enhanced by a gp120 N-terminal deletion. *J Virol*. 2013 Feb.87:1554. [PubMed: 23175357]
59. Montefiori DC, et al. Magnitude and breadth of the neutralizing antibody response in the RV144 and Vax003 HIV-1 vaccine efficacy trials. *J Infect Dis*. 2012 Aug.206:431. [PubMed: 22634875]
60. Gray ES, et al. Broad neutralization of human immunodeficiency virus type 1 mediated by plasma antibodies against the gp41 membrane proximal external region. *J Virol*. 2009 Nov.83:11265. [PubMed: 19692477]
61. Pollara J, et al. High-throughput quantitative analysis of HIV-1 and SIV-specific ADCC-mediating antibody responses. *Cytometry A*. 2011 Aug.79:603. [PubMed: 21735545]
62. Bonsignori M, et al. Antibody-dependent cellular cytotoxicity-mediating antibodies from an HIV-1 vaccine efficacy trial target multiple epitopes and preferentially use the VH1 gene family. *J Virol*. 2012 Nov.86:11521. [PubMed: 22896626]
63. Liu P, et al. Infectious virion capture by HIV-1 gp120-specific IgG from RV144 vaccinees. *J Virol*. 2013 Jul.87:7828. [PubMed: 23658446]
64. Smith K, et al. Rapid generation of fully human monoclonal antibodies specific to a vaccinating antigen. *Nat Protoc*. 2009; 4:372. [PubMed: 19247287]
65. Ewing B, Green P. Base-calling of automated sequencer traces using phred. II. Error probabilities. *Genome Res*. 1998 Mar.8:186. [PubMed: 9521922]
66. Ewing B, Hillier L, Wendl MC, Green P. Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Res*. 1998 Mar.8:175. [PubMed: 9521921]
67. Kepler TB, et al. Chiropteran types I and II interferon genes inferred from genome sequencing traces by a statistical gene-family assembler. *BMC Genomics*. 2010; 11:444. [PubMed: 20663124]
68. Smith TF, Waterman MS. Identification of common molecular subsequences. *J Mol Biol*. 1981 Mar.147:195. [PubMed: 7265238]
69. Volpe JM, Cowell LG, Kepler TB. SoDA: implementation of a 3D alignment algorithm for inference of antigen receptor recombinations. *Bioinformatics*. 2006 Feb.22:438. [PubMed: 16357034]
70. Bonsignori M, et al. Analysis of a clonal lineage of HIV-1 envelope V2/V3 conformational epitope-specific broadly neutralizing antibodies and their inferred unmutated common ancestors. *J Virol*. 2011 Oct.85:9998. [PubMed: 21795340]
71. Liao HX, et al. Co-evolution of a broadly neutralizing HIV-1 antibody and founder virus. *Nature*. 2013 Apr.496:469. [PubMed: 23552890]
72. Kepler TB, et al. Reconstructing a B-Cell Clonal Lineage. II. Mutation, Selection, and Affinity Maturation. *Front Immunol*. 2014; 5:170. [PubMed: 24795717]

Figure 1. Characteristics of HIV-1-reactive antibodies (Abs) induced by DNA prime, rAd5 boost vaccine

A) Plasma Ab titers (AUC, area under the curve) to gp120 and gp41 proteins in a subset of 40 HVTN 505 vaccine recipients; red circles represent vaccine responders (percents) to the antigens tested, and blue represent non-responders. (B) Plasma binding Abs from 8 HVTN 082 and 204 trial participants (vaccine responders) were screened for gp120 and gp41 reactivity via binding Ab multiplex assay (BAMA). (C) HIV-1-reactive Abs were isolated from single memory B cells of the 8 HVTN 082 and 204 trial participants and screened for binding gp120 and gp41 proteins via ELISA. Statistics: (A) *** $p < 0.0001$, Wilcoxon

signed rank test, B.MN gp41 vs. gp120 (maximum; which is the highest value of all gp120 Envs for each participant), $*p < 0.05$ McNemar's test, B.MN gp41 vs. each gp120 (not shown); (B) $*p < 0.05$ $**p < 0.01$, Wilcoxon signed rank test [Graphpad Prism].

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 2. Polyreactivity of vaccine-induced gp41-reactive monoclonal antibodies (mAbs)
 Five representative polyreactive and gp41-reactive mAbs demonstrated binding with auto-antigens (ANA) (A), Hep-2 cells (immunofluorescence staining) (B), and anaerobe and aerobe whole cell lysates of intestinal microbiota (IM), bacterial (*E. coli*) RNA polymerase, MN gp41, and vaccine-strain VRC-A gp140 antigens (BAMA) (C). (B) All images were taken on an Olympus AX70 fluorescence microscope using a 40x objective with a SPOT Flex camera. All images were prepared using 50 $\mu\text{g/mL}$ of mAb. Images were acquired for 5 sec (2F5, 17b) or 10 sec (DH432, DH438, DH440, DH443, DH444). Size bars are 25 μm .

(A, B) Control antibodies: 4E10 (gp41), 2F5 (gp41), 17b (gp41), and palivizumab (RSV).
(C) MAb binding was reported as MFI-background-blank, where background is plate specific background binding and blank represents non-specific sample binding to a negative control bead. Positivity cutoff for binding was 100 MFI as previously reported (2); AbCLL1324 as an IM-reactive control was used to validate IM binding and gp41 mAb 7B2 that does not react with IM was used as a negative control. Data shown were generated from the same commercially available kits (A, B), and a single BAMA experiment (C), which was in agreement with Ab-IM reactivity in western blots (Figure S10 or not shown).

A. Immunogenetics

PTID	HVTN Trial	Vaccination timepoint	mAb ID	VH	DH	JH	VH mutation frequency (%), nt	HCDR3 length, aa	Ig Isotype
082-003	082	PRE	DH477	1-69*06	3-9*01	4-1*02	0	11	M
		POST	DH476	1-69*06	3-9*01	4-1*02	5.3	11	G

B. DH477/DH476_VH DJH junctions

C. SPR analysis of DH477 and DH476 binding profile

D. DH477/DH476 Antibody binding kinetics

HIV-1 Envelopes	DH477 (pre-vaccine)			DH476 (post-vaccine)		
	k _{on} , M ⁻¹ s ⁻¹	k _{off} , s ⁻¹	K _d , nM	k _{on} , M ⁻¹ s ⁻¹	k _{off} , s ⁻¹	K _d , nM
A.92RW020	nb	nb	nb	13x10 ⁴	4x10 ⁻⁴	3
B.HxB2/Bal-V3	nb	nb	nb	20x10 ⁴	3x10 ⁻⁴	2
C.97ZA012	nb	nb	nb	28x10 ⁴	34x10 ⁻⁴	12
M.CON-S	nb	nb	nb	32x10 ⁴	10x10 ⁻⁴	3
MN gp41	---	---	41*	10x10 ³	7x10 ⁻⁵	7
gp41 5-helix	---	---	21*	2x10 ⁵	2x10 ⁻⁴	1

*K_d determined by steady state analysis. nb, no binding.

E. DH477 binding of VRC-A gp145 Env

F. DH477/DH476 Cross-reactivity profile

Figure 3. Characteristics of clonally-related antibodies (Abs)

Pre- (DH477) and post- (DH476) vaccine clonally-related Abs found in vaccinee 082-003 had the same V_HDJ_H recombination. IGHV, IGHD and IGHJ Ab segments were statistically inferred (A, B); nucleotides in the 3' end of the V-gene and 5' end of J-gene shown with rearrangement junctions (gray-shaded nucleotides - NN1, NN2), and HCDR3 (underlined nucleotides) indicated. The lower case letters show nucleotides that have been removed from the germline gene during V_HDJ_H rearrangement. Pre- (DH477) and post- (DH476) vaccine heavy chain sequences were paired with the natural light chain (IGκV3-20*01, IGκJ1*01,

1.7% mutated nt, 10 aa CDR3) of the post-vaccine Ab DH476. (C) Ab binding to recombinant HIV-1 Env gp140, 5-Helix gp41 and MN gp41 proteins was determined by surface plasmon resonance (SPR) analysis, and (D) the antibody binding K_d determined by rate constants or steady-state analysis measurements. (E) Binding of DH477 to VRC-A gp140 expressed on the surface of 293i cells. Anti-V3 mAb (19B) and a representative gp41-reactive Ab (DH440) from this study, both of which bind recombinant VRC-A gp140, were used as positive controls, while palivizumab (RSV Ab) was used as a negative IgG1 control. (F) The cross-reactivity of pre-(DH477) and post-(DH476) vaccine Abs with intestinal microbiota (IM) whole cell lysates (WCLs) was determined via western blot and SPR as previously described (2). DH438 (gp41-reactive Ab) and palivizumab were used as positive and negative control Abs, respectively, for western blots, and the arrows indicate candidate IM antigens bound by DH476 and/or DH477. In SPR analysis, IM WCLs were diluted in PBS buffer as indicated and injected over mAbs captured on an anti-IgFc immobilized sensor surface. Specific binding of the lysate proteins to the mAbs in SPR were measured after subtraction of non-specific binding to anti-HA mAb CH65. Abbreviations - nt, nucleotides; aa, amino acid. Data shown (C–F) were representative of duplicate experiments, except SPR screen of DH477 and DH476 for binding rgp140 proteins that was performed once (C).

Table 1
Frequency of 54L and 54F-HCDR2 bearing IGHV1-69-using antibodies induced by HIV and Influenza

Cohort	Antibody reactivity	Total Antibodies	IGHV1-69 Abs	HCDR2-54L	HCDR2-54F	Statistics; HCDR2_54L:54F
HIV-1 Vaccination ¹	gp41	205	125/205 (61%)	116/125 (93%)	9/125 (7%)	$p < 0.45^{(1,2)}$
HIV-1 Infection ²	gp41	116	42/116 (36%)	41/42 (98%)	1/42 (2%)	
HIV-1 Infection ³	Non HIV-1	971	64/971 (7%)	18/64 (28%)	46/64 (72%)	$p < 0.0001^{(1,3,2,3)}$
Influenza Infection or Vaccination ⁴	Hemagglutinin ⁵	278	10/278 (4%)	1/10 (10%)	9/10 (90%)	$p < 0.0001^{(1,4,2,4)}$; $p = 0.44^{(3,4)}$

Counts reflect number of unique antibody (Ab) heavy chain sequences containing full VHDJH rearrangements without stop codons.

¹ 8 HVTN 204 and 082 trial participants
^{2,3} 29 HIV-1 infected subjects (2, 3, 23)
⁴ 13 influenza infected or vaccinated subjects (24)
⁵ Influenza antibody specificity as measured by reactivity with purified recombinant HA-enriched trivalent influenza vaccine (Fluzone). Statistics performed using Fisher's Exact Test, SAS

Table 2

Polyreactivity of DNA prime, rAd5 boost vaccine-induced gp41-reactive and gp120-reactive monoclonal antibodies

Antigens	gp41-reactive antibodies	gp120-reactive antibodies	<i>p</i> -value ⁷
Anaerobe-intestinal microbiota ¹	14/17 (82%)	8/12 (67%)	0.40
Aerobe-intestinal microbiota ²	13/17 (76%)	5/12 (42%)	0.12
Bacterial (<i>E. coli</i>) RNA Polymerase ³	9/17 (53%)	2/12 (17%)	0.06
AtheNA auto-antigen panel ⁴	11/17 (65%)	4/12 (33%)	0.14
HEp-2 cells (IFA) ⁵	5/17 (29%)	1/12 (8%)	0.35
Cardiolipin ⁶	4/17 (24%)	1/12 (8%)	0.63
All antigens tested, gp41-reactive vs. gp120-reactive antibodies			0.003 ⁸

Antibodies (Abs) were screened for cross-reactivity with anaerobe and aerobe whole cell lysates of intestinal microbiota from human stool^{1,2} and recombinant bacterial (*E. coli*) RNA polymerase³ (Binding Ab multiplex assay; BAMA), host antigens (AtheNA)⁴, HEp-2 cells (immunofluorescence assay, Zeuss Scientific)⁵, and cardiolipin (QUANTA Lite[®] ACA IgG III)⁶. *p*-value generated via Fisher's Exact Test (SAS v9.3)⁷ or Cochran-Mantel-Haenszel Test controlling for Antigen (SAS v9.3)⁸