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Polyfunctional Fc-Effector Profiles Mediated by IgG Subclass Selection Distinguish RV144 and VAX003 Vaccines

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The human phase 2B RV144 ALVAC-HIV vCP1521/AIDSVAX B/E vaccine trial, held in Thailand, resulted in an estimated 31.2% efficacy against HIV infection. By contrast, vaccination with VAX003 (consisting of only AIDSVAX B/E) was not protective. Because protection within RV144 was observed in the absence of neutralizing antibody activity or cytotoxic T cell responses, we speculated that the specificity or qualitative differences in Fc-effector profiles of nonneutralizing antibodies may have accounted for the efficacy differences observed between the two trials. We show that the RV144 regimen elicited nonneutralizing antibodies with highly coordinated Fc-mediated effector responses through the selective induction of highly functional immunoglobulin G3 (IgG3). By contrast, VAX003 elicited monofunctional antibody responses influenced by IgG4 selection, which was promoted by repeated AIDSVAX B/E protein boosts. Moreover, only RV144 induced IgG1 and IgG3 antibodies targeting the crown of the HIV envelope V2 loop, albeit with limited coverage of breakthrough viral sequences. These data suggest that subclass selection differences associated with coordinated humoral functional responses targeting strain-specific protective V2 loop epitopes may underlie differences in vaccine efficacy observed between these two vaccine trials.

INTRODUCTION

The RV144 phase 2B HIV vaccine trial (ALVAC-HIV vCP1521 prime and recombinant gp120 AIDSVAX B/E boost) was the first HIV vaccine to exhibit reduced risk of infection among vaccinees (*1*). Whereas vaccine efficacy was modest, knowledge could be engendered from the comparison of this potential protective vaccine with vaccines demonstrating no protective efficacy. One component of the RV144 vaccine, the AIDSVAX B/E bivalent rgp120 protein, was also given with alum in seven doses in the VAX003 phase 3 vaccine trial; however, alone, the AIDSVAX B/E did not provide any protection from infection in intravenous drug users (*2*). Both vaccines induced gp120-specific antibodies (Abs), but only limited neutralizing Ab activity (*3*–*6*) and negligible cytotoxic T cell responses (*7*). Thus, it is plausible that differences in vaccine efficacy may be attributable to other antiviral properties of the humoral immune response.

The follow-up analysis of the immune correlates of risk in the RV144 trial identified two features of the vaccine-induced immune response that were associated with differential risk of infection among vaccinees, including a negative effect of vaccine-induced immunoglobulin A (IgA) responses and a beneficial effect of Ab responses targeting the V1/V2 region of the HIV envelope (*8*). Moreover, in the absence of IgA responses, Ab-dependent cellular cytotoxicity (ADCC) was associated with a reduced risk of infection (*8*), suggesting that Ab functions beyond neutralization may play a critical role in immunity to HIV. To further gain a mechanistic appreciation of the impact of the V1/V2 Abs, viral sieve analyses pointed to a vaccine-induced selection of viral variants in the crown of the V2 loop, directly implicating V2-specific Abs in strain-specific, vaccine-mediated protective efficacy (*9*). Yet, collectively, the mechanisms by which these vaccine-specific Abs conferred protection and how to optimally induce such responses remain unclear.

Beyond neutralization, Abs mediate additional antiviral activities, including cellular cytotoxicity, phagocytosis, and viral inhibition (*10*), through their capacity to recruit the antiviral activity of the innate immune system via Fc receptors. However, little is known regarding how these functions are immunologically coordinated and how vaccines may direct these Ab effector functions. We hypothesized that the RV144 and VAX003 trials elicited different humoral functional profiles that could provide key insights into the mechanism by which immunization strategies skew humoral immunity. Here, we show that RV144 elicited a polyfunctional Ab response associated with the selective induction of IgG3 Abs. By contrast, VAX003 elicited a monofunctional Ab response strongly influenced by the selection of the more functionally inert IgG4 Ab subclass. This skewed IgG4 subclass profile was promoted by repeated administration of gp120 protein vaccination, marked by a concomitant decrease in IgG3 subclass levels and alterations in Ab functionality with repeated protein exposure. Moreover, whereas VAX003 induced a highly focused IgG4 Ab response against the crown of the V2 loop, the RV144 vaccine induced both

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IgG1 and IgG3 Abs targeting this region, preferentially targeting strains blocked by the vaccine but with limited coverage of breakthrough sequences. Thus, both trials induced suboptimal humoral immune profiles, but qualitative analyses probing beyond Ab titer and neutralization indicate that selection of functional Ab subclasses that target vulnerable envelope regions may be potent contributors to vaccine efficacy.

RESULTS

RV144 induces qualitatively different polyfunctional Ab responses

VAX003 induced higher gp120 binding titers (*1*, *2*) (fig. S1, A to C) and consequently linked higher Fc-effector profiles compared to RV144 (fig. S1, D to I), suggesting that neither Ab titer nor Ab function alone could account for the reduced risk of infection observed in the RV144 trial. Therefore, we sought to determine whether there were qualitative differences in nonneutralizing Fc-effector profiles between the two vaccine trials. It is possible that Abs able to mediate multiple functions, for example, ADCC, Ab-dependent cellular phagocytosis (ADCP), or Ab-mediated release of cytokines/chemokines, or specific combinations of functions, may afford greater protection via their ability to harness a broader range of innate antiviral functions available in distinct tissue compartments. Thus, we evaluated the relationship between six functional variables [ADCC, ADCP, CD107a degranulation, interferon-γ (IFN-γ), macrophage inflammatory protein-1β (MIP-1β) secretion, and polyfunctional natural killer (NK) activation] from IgG purified from plasma samples taken 2 weeks after final vaccination for both RV144 and VAX003 vaccines. Because gp120MN- and gp120A244-specific titers and function were highly correlated (fig. S2), full functional profiling was performed with only gp120MN. Positive correlations were observed between most of the functional parameters measured in RV144 (Fig. 1A), suggesting the induction of polyfunctional humoral responses. By contrast, weaker or no correlations or inverse correlations were observed in the VAX003 trial (Fig. 1B), suggesting that in a given VAX003 vaccinee, Abs had a more restricted effector profile and were rarely able to elicit multiple functions. Thus, RV144 induced a coordinated polyfunctional Ab response compared to VAX003 as reflected by higher correlation coefficients among functions (fig. S3).

RV144 and VAX003 promoted distinct Ab subclass profiles

IgG1 and IgG3 exhibit higher Fc receptor affinity and therefore recruit innate immune effector activity more readily compared to IgG2 and IgG4, which are comparatively weaker recruiters of Fc receptor–mediated cytolytic activity (*10*, *11*). Moreover, in plasma, IgG1 and IgG2 levels greatly exceed IgG3 and IgG4 levels (*12*). Therefore, we next aimed to determine whether subclass selection differences could account for the distinct functional profiles observed among the vaccine recipients. Overall, both vaccines predominantly elicited gp120-specific IgG1 Abs (Fig. 2A), albeit at lower levels in RV144 compared to VAX003 (Fig. 2B). However, VAX003 elicited IgG2 and IgG4 Abs in a larger proportion of vaccinees compared to RV144 (Fig. 2A) and at higher total levels compared to RV144 (Fig. 2, C and E). By contrast, RV144 selectively induced gp120-specific IgG3 Abs in 58% of vaccinees as compared with 38% of VAX003 vaccinees, and total IgG3 levels were 4.6 times greater in RV144 vaccinees compared with VAX003

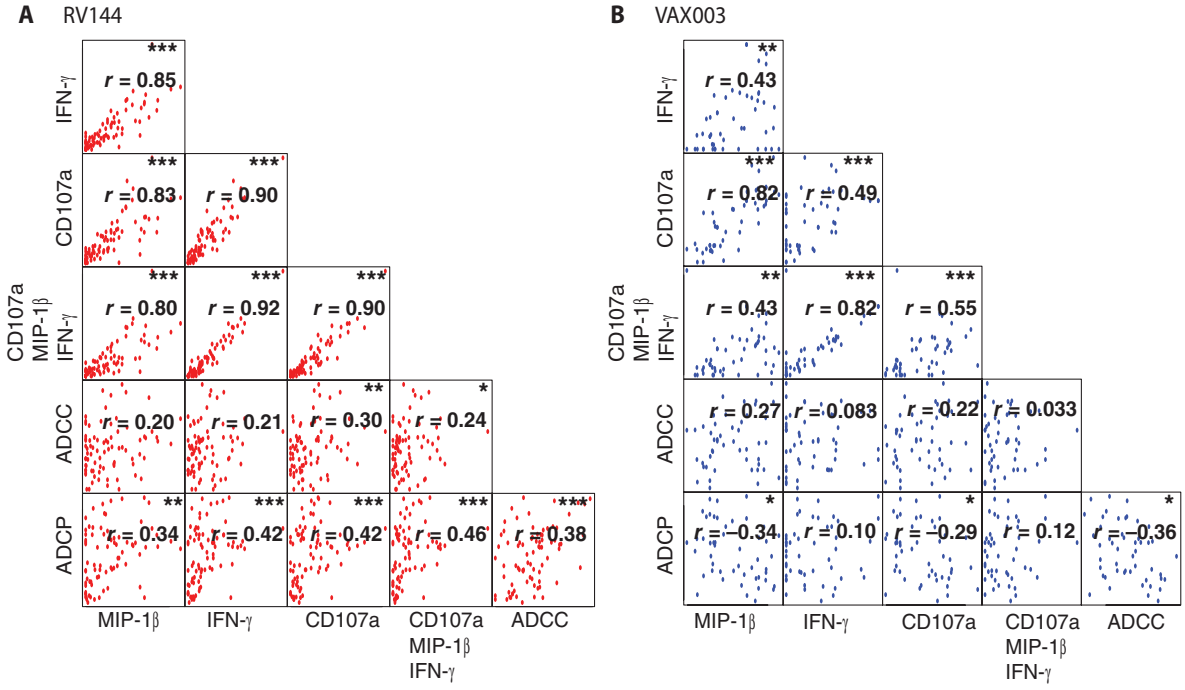

Fig. 1. Ab function correlation matrices for RV144 and VAX003. (**A** and **B**) Scatter plot matrices represent the relationship between Fc-mediated effector functions including NK cell–mediated MIP-1β, IFN-γ, CD107a, MIP-1β/IFN-γ/CD107a$^+$, ADCC, or ADCP (plotted along both *x* and *y* axes) for (A) RV144 and (B) VAX003. *r* values represent Spearman rho. ***$P < 0.0001$, **$P < 0.01$, *$P < 0.05$.

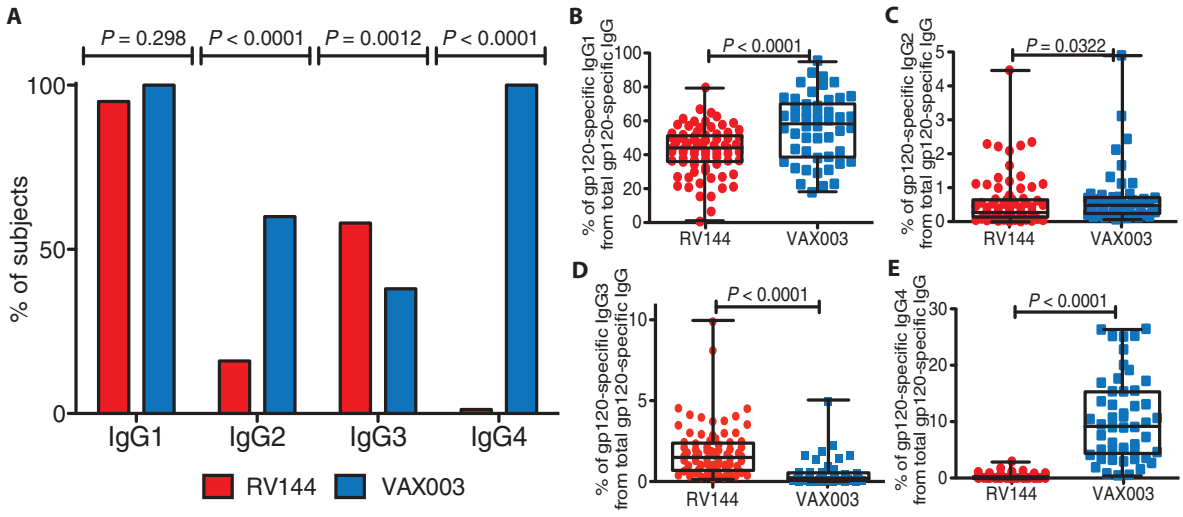

Fig. 2. gp120-specific IgG subclass selection in the RV144 and VAX003 trials. A customized Luminex subclass assay was conducted using purified bulk IgG isolated from RV144 and VAX003 samples to determine the levels of gp120-specific IgG1, IgG2, IgG3, and IgG4 Abs. A positive threshold was determined for each subclass by assaying 20 RV144 and 10 VAX003 placebo samples and determining median fluorescence intensity (MFI) + 2 SDs for each subclass to ensure a 95% confidence interval. (**A**) The frequency of subjects positive for each of the IgG subclasses is depicted in the bar graph for RV144 (red) and VAX003 (blue). *P* values were calculated by Fisher's exact test. (**B** to **E**) Differences in the overall quantity of each subclass are depicted in the dot plots for (B) IgG1, (C) IgG2, (D) IgG3, and (E) IgG4 for each vaccine trial, based on the respective % IgG subclass MFI from total HIV-specific IgG MFI. Differences in isotype frequencies between the two trials were calculated by Mann-Whitney test.

vaccinees (Fig. 2, A and D). Thus, RV144 vaccination uniquely induced higher levels of IgG3 Abs compared to VAX003 marked by higher levels of gp120-specific IgG1s and functionally inferior IgG2/IgG4 Abs.

IgG3 levels predict enhanced functionality for the RV144 vaccine

To next determine whether changes in Ab subclass selection between the two trials were associated with differences in Ab functionality, we interrogated the relationships between subclass levels and functionality. Remarkably, only HIV-specific IgG1 levels predicted ADCC activity in VAX003 (Fig. 3B), suggesting that other humoral parameters beyond subclass selection likely govern different Ab functional activities among vaccinees. Conversely, for both VAX003 and RV144 (Fig. 3, A and B), IgG2 and IgG4 subclass levels were inversely correlated with functional activity, suggesting a potential inhibitory role for these isotypes. Conversely, HIV-specific IgG1 and IgG3 levels clearly predicted nearly all functions in the RV144 vaccine trial (Fig. 3A). However, because vaccine-specific IgG1 and IgG3 were induced in parallel within RV144 samples (fig. S4A) and IgG3 was the only Ab subclass that uniquely segregated with RV144 compared to VAX003 vaccinees (Fig. 3A), these data suggested an important role for this quantitatively minor, but functionally superior, IgG3 Ab subclass in driving functionally coordinated Ab activity in RV144 vaccinees.

Vaccine-elicited IgG3 Abs enhance Ab effector function, whereas IgG4 Abs inhibit Ab effector function

Because IgG3 Abs harbor the highest functional activity among all the subclasses (*13*, *14*) and were selectively induced in RV144 compared to VAX003 vaccinees (Fig. 3A), we next sought to determine whether these Abs were the critical vaccine-induced Abs involved in modulating Ab functionality. Therefore, IgG3 Abs were depleted from a set of 16 RV144 and VAX003 samples with moderate activity. Depletion of IgG3 from the RV144 samples resulted in a significant reduction in ADCP activity ($P < 0.0001$, Fig. 4A) and significant loss of ADCC activity ($P = 0.019$, Fig. 4B) when compared with undepleted bulk IgG. Similarly, there was a significant decrease in ADCP activity in VAX003 samples ($P = 0.022$, Fig. 4A), and a trend but insignificant decrease in ADCC (Fig. 4B). Overall, these results confirm that despite IgG3 Abs representing a minor fraction of total circulating Abs, depletion of this subclass resulted in a profound diminution of Ab functionality in both trials, emphasizing the functional importance of IgG3 Abs in mediating both ADCC and ADCP (that is, polyfunctional Ab activity) in RV144 vaccinees and ADCP in VAX003 vaccinees. In addition, before depletion, there was a trend toward a positive relationship between all Fc-functional activities in RV144 samples ($r = 0.52$, $P = 0.06$, fig. S5A), which was lost upon IgG3 depletion. By contrast, no polyfunctional Fc-effector relationship was observed in VAX003 samples either before or after IgG3 depletion (fig. S5B), confirming that IgG3 Abs drive coordinated Ab functional profiles in RV144.

Because VAX003 vaccination induced higher levels of IgG4, which are classically associated with lower functionality, we next aimed to determine whether the induction of IgG4 Abs was simply induced in association with an uncoordinated functional response or potentially contributed directly to the poorly coordinated response. Thus, IgG4 Abs were depleted from a set of 16 functionally matched samples across both trials. A significant increase in ADCP activity ($P = 0.0006$, Fig. 4C) and a trend toward increased ADCC ($P = 0.066$, Fig. 4D) was observed for VAX003 samples compared to bulk IgG. By contrast, there was no significant difference observed between bulk or IgG4-depleted RV144 vaccine samples, likely due to the low levels of this subclass among RV144 vaccine–specific responses. These data suggest that IgG4 Abs are not simply associated with the induction of an uncoordinated Ab response but may directly inhibit Ab Fc-effector

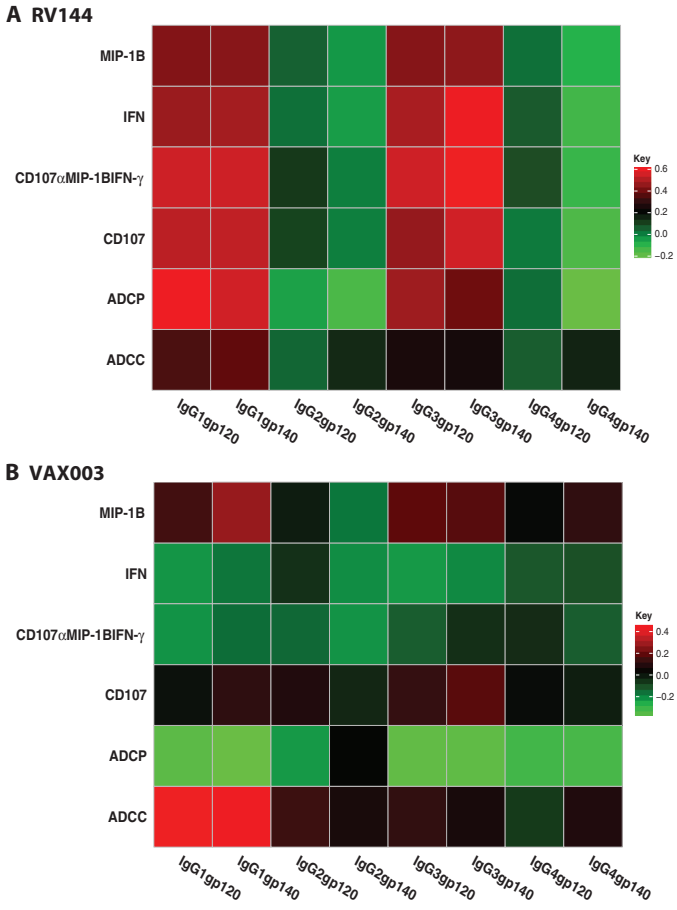

Fig. 3. Ab subclass selection impacts Fc-mediated function. (**A** and **B**) Heat maps show the Spearman rank correlation coefficients between gp120- or gp140-specific IgG subclass levels and function for (A) RV144 and (B) VAX003. The color represents the strength and direction of the correlation. Red represents a positive correlation, black represents no correlation, and green represents a negative correlation.

function. Intriguingly, in contrast to IgG3 depletion, depletion of IgG4, however, did not alter the coordination of polyfunctional Ab activity (fig. S5D).

Boosting altered Ab subclass selection in VAX003

Although both RV144 and VAX003 vaccine regimens included the same recombinant gp120 proteins, the RV144 vaccine regimen delivered four viral vector vaccinations, the last two vaccinations accompanied by protein boosts, whereas the VAX003 vaccine regimen consisted of seven rounds of protein immunization (*1*, *2*). Therefore, we next aimed to determine whether functional differences observed between the two vaccine strategies at the end of each vaccine regimen were already present at time points when similar numbers of protein boosts (*2*) had been administered and if the functional profiles changed after additional protein vaccinations in VAX003. Therefore, Fc-effector functional profiles, along with gp120-specific IgG isotype subclass profiles of VAX003 samples from post-second (VAX003 visit 5) and post-third protein vaccination (VAX003 visit 6), were analyzed and compared to final RV144 and VAX003 (visit 15) vaccination time points.

Weaker ADCC activity (ninefold weaker than the final VAX003 time point and fivefold weaker compared to RV144) (Fig. 5A) was observed at VAX003 visit 5, whereas no difference in ADCP activity was observed compared to RV144 responses (Fig. 5B). This reduced ADCC activity was observed despite equivalent gp120-specific Ab titers in RV144 and VAX003 visit 5 (Fig. 5C), suggesting that a qualitative difference in functional activity was already programmed after only two boosts, potentially in the setting of the two canary pox primes. This functional difference was associated with lower IgG1 levels (Fig. 5D) but elevated levels of IgG2 and IgG3 Ab subclasses at VAX003 visit 5 compared to RV144 (Fig. 5, E and F). These surprisingly high levels of IgG3 likely drove a strong ADCP response (fig. S6D), but not ADCC activity, suggesting that protein boosting alone can drive IgG3 subclass selection and that viral vector priming alone could not explain this unique Ab subclass selection profile. By contrast, IgG4 levels remained low after only two boosts at VAX003 visit 5 (Fig. 5G). After one additional boost (VAX003 visit 7), overall gp120-specific titers increased robustly (twofold over VAX003 visit 5, Fig. 5C), linked to a significant increase in ADCC ($P = 0.033$) but no difference in ADCP. ADCP and ADCC activities at both VAX003 visits 5 and 7 (Fig. 5, H and I) were correlated to one another, but this relationship was completely lost and even inversed with increased boosting at visit 15 (Fig. 5J). This inverted relationship was linked to marked changes in the Ab subclass repertoire selection, associated with increasing IgG1 and IgG4 levels (Fig. 5, D and G) but significantly declining IgG3 levels ($P = 0.011$, Fig. 5I). These data suggest that alterations in Ab subclass distribution, in the setting of overall boost-induced total gp120-specific Ab titer increases, were linked to alterations in Ab functionality in VAX003.

Subclass selection biases may contribute to virus breakthrough

The immune-correlates analysis of the RV144 vaccine trial indicated that binding of IgG Abs to variable regions 1 and 2 (V1/V2) of HIV-1 gp120 correlated inversely with HIV-1 infection (*8*). However, whether specific Ab subclasses were induced against this region is unknown. Whereas no difference was observed in the gp70V1/V2-specific IgG2 Abs among the trials, higher IgG1 and IgG4 levels were observed in the VAX003 vaccinees (Fig. 6A). By contrast, the RV144 vaccinees again selectively induced significantly higher proportions of gp70V1/V2-specific IgG3 Abs ($P < 0.0001$, Fig. 6C). These data suggest that subclass selection biases observed in the bulk gp120-specific Ab pool were mirrored in the gp70V1/V2-specific Ab pool.

Previous mapping of the gp70V1/V2 region showed predominant targeting of the crown of the V2 loop (*15*) (amino acids 167 to 181), and sequence analysis of breakthrough viruses demonstrated that the vaccine selectively blocked infections with HIV-1 variants with mutations at positions 169 and 181 in V2 (*9*). In contrast to bulk gp120- and gp70V1/V2-specific Ab responses, RV144 induced higher IgG1 responses against all the V2 variants compared to VAX003 vaccinees (Fig. 6, E to H). Consistent with previous findings, RV144 vaccination was associated with a skewed and robust induction of IgG3 Abs (Fig. 6, M to P), whereas VAX003 only induced high levels of IgG4 Abs targeting this region (Fig. 6, Q to T), suggesting that VAX003 immunization induced negligible functional Ab responses covering the crown of the V2 loop.

Because mutations at position K169 and maintenance of an I181 were associated with increased risk of infection in RV144 vaccinees (*9*), consensus and mutant peptide–specific subclass distribution was assessed. RV144 induced significantly lower IgG1 and IgG3 levels rec-

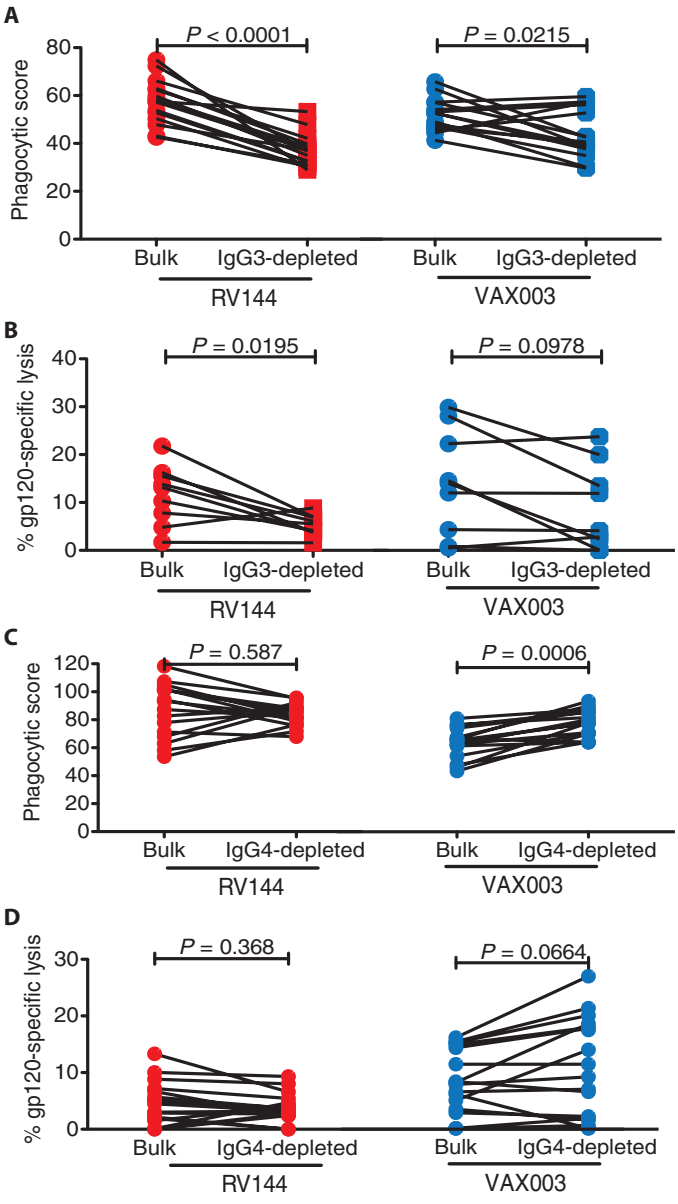

Fig. 4. Vaccine-elicited IgG3 Abs increase Fc-effector function, whereas IgG4 Abs inhibit effector function. IgG3 and IgG4, respectively, were depleted from bulk purified IgG of RV144 and VAX003 samples using IgG3/IgG4 capture beads. (**A** and **B**) The IgG3-depleted and original bulk purified IgG were then tested for ADCP (A) and ADCC activity (B). (**C** and **D**) Similarly, IgG4-depleted and bulk purified IgG responses were also tested for ADCP (C) and ADCC activity (D). RV144 samples are identified by red symbols; VAX003 samples are identified by blue symbols. *P* values were calculated by Wilcoxon signed-rank tests.

ognizing the K169Q variant when compared with the consensus sequence (IgG1, $P = 0.0005$; IgG3, $P = 0.0023$) or the I181L variants (IgG1, $P = 0.0003$; IgG3, $P = 0.02$) (Fig. 6, E, F, M, and N), paralleling the reduced ability of the vaccine to block viruses with the K169Q mutation, associated with reduced sensitivity to neutralization (*16*–*18*). By contrast, RV144 induced high levels of IgG1 and IgG3s recognizing the double K169Q/I181L peptide (92TH023_VCP1521/CM244 background) (Fig. 6, F and N), corresponding to the HIV-1 variant most likely to be blocked in the RV144 trial. The presence of K169Q dominated the I181X effect (Fig. 6, E, F, M, and N), although, on the 92TH023 background, Ab recognition of the K169Q variant was profoundly modulated by variation at position 181. Overall, these data suggest that the RV144 vaccine was preferentially able to induce highly functional IgG1 and IgG3 Abs against consensus and I181X viruses, the variants selectively blocked by the RV144 vaccine, but may have been poorly protective against breakthrough viruses because of limited Ab breadth targeting the highly variable V2 loop.

DISCUSSION

Although the protective efficacy of the RV144 vaccine trial has been intensely scrutinized, the immune-correlates (*8*) and follow-up analyses (*6*, *9*, *19*, *20*) have begun to shed critical light on the unique immunological signatures associated with this vaccine. Beyond the differences observed among the RV144 case/controls, we speculated that a comparative analysis of Ab functionality with Abs elicited in the VAX003 trial, which used the same recombinant protein immunization in a Thai population, could reveal critical nuances in the vaccine-elicited immune response induced by two different vaccine strategies. Specifically, we hypothesized that important information could be garnered regarding the impact of a viral vector prime/boost versus a protein-alone immunization regimen that collectively may point to immune mechanisms that may underlie the differences in observed vaccine efficacy between the two trials. Our data suggest that titer alone may not inform vaccine efficacy. Rather, an "effective titer," which moves beyond neutralization and binding titers alone to take antigen-specific Ab functionality and subclass selection into account, may provide a more informative measure of protective vaccine activity.

At the end of the vaccine regimens, HIV-specific Abs were induced by both trials; however, RV144 induced lower titers than VAX003 (fig. S1C). Yet, RV144 induced a polyfunctional Ab profile that was able to simultaneously recruit multiple effector functions in a coordinated manner, whereas VAX003 induced Abs with either NK cell–recruiting or phagocytic activity, but rarely both. Although it is uncertain whether ADCP, NK cell activation, or ADCC is required for protection from infection, it is possible that by interacting with a wider range of innate immune cells that may be present in different amounts and combinations at various transmission sites, polyfunctional Abs may simply provide a higher probability of protection from infection.

Subclass selection represents one of the key Ab features exploited during the design of monoclonal therapeutics (*21*, *22*) to potentiate/impair Ab function. Of the four IgG subclasses, IgG1 and IgG3 are associated with higher affinities for Fcγ receptors (*12*) and, consequently, stronger activation of Fc-mediated effector functions. By contrast, IgG2 and IgG4 have weaker affinities for Fcγ receptors, with IgG4 having 10-fold weaker affinity than IgG1 (*23*). Malaria-specific immunity has been associated with skewed subclass selection, with IgG1/IgG3 selection associated with immunity and IgG2-biased responses associated with the lack of protection (*24*–*26*). Likewise, Ab functionality was closely linked to distinct subclass profiles in RV144 and VAX003 vaccinees. When compared with VAX003, RV144 induced higher levels of IgG3 Abs compared to VAX004, whereas VAX003 promoted higher levels of IgG1, IgG2, and IgG4 Ab levels. Moreover, longitudinal analysis of alum-adjuvanted VAX003 demonstrated an expected IgG1-biased re-

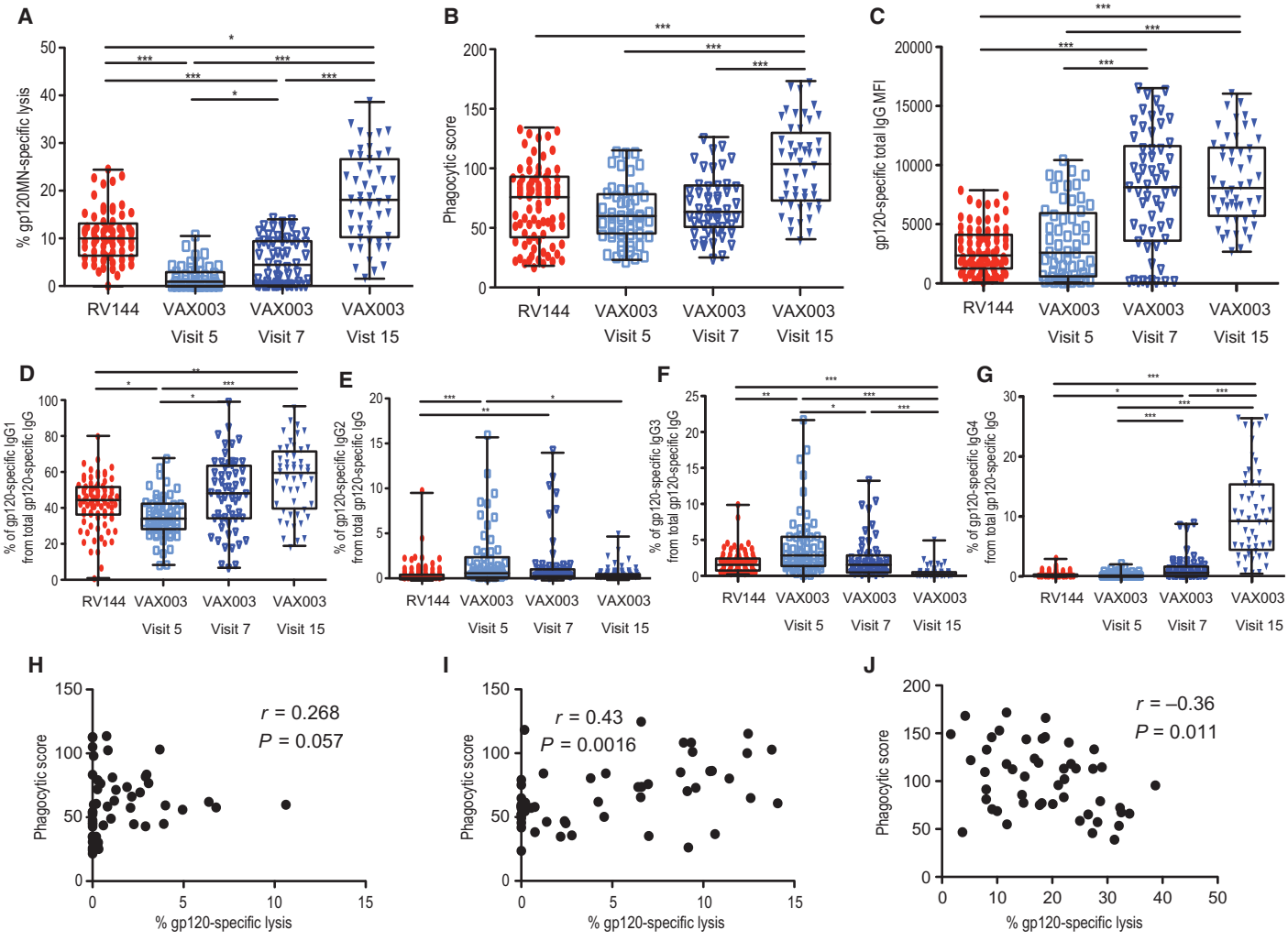

Fig. 5. Impact of boosting on subclass selection and Ab function. (**A** and **B**) VAX003 samples after the second (VAX003 visit 5) and after the third protein vaccination (VAX003 visit 7) were tested for (A) ADCC and (B) ADCP activity at 100 μg/ml and compared to RV144 and VAX003 visit 15 (both 2 weeks after final vaccination). (**C** to **G**) gp120-specific titers for (C) total IgG as well as (D) IgG1, (E) IgG2, (F) IgG3, and (G) IgG4, based on respective gp120-specific IgG subclass MFI/total gp120-specific IgG MFI. (**H** to **J**) Scatter plots of ADCP versus ADCC activity for (H) VAX003 visit 5, (I) VAX003 visit 7, and (J) VAX003 visit 15. *P* values were determined by Kruskal-Wallis tests with Dunn's post hoc analyses. ***$P < 0.0001$, **$P < 0.01$, *$P < 0.05$.

sponse with increased titers and functionality after repeated boosting. However, boosting simultaneously resulted in reduced polyfunctionality linked to the concomitant induction of IgG4 responses. In melanoma, both antigen-specific and bulk IgG4 Abs have been shown to inhibit Ab-guided tumoricidal activity by reducing Fc receptor activation (*22*). Previous studies in the VAX004 trial also showed an antagonistic effect of gp120-specific IgG2 Abs (*27*). Likewise, depletion of IgG4 Abs from VAX003, where some individuals induced HIV-specific IgG responses consisting of up to 30% IgG4, resulted in significant increases in Fc-mediated effector functions, supporting an antagonistic role for IgG4 Abs in VAX003.

The uncharacteristic induction of high levels of IgG4 in the alum-based VAX003 regimen may be related to the repeated administration of seven large doses of vaccine antigens in the absence of sufficiently potent adjuvant signals that may have driven excessive B cell receptor triggering. Allergy studies have similarly observed that continuous exposure to high doses of antigen can result in suppressed inflammatory responses through elevated levels of antigen-specific IgG4 (*28*). However, little is known about the mechanism by which vaccines tune Ab subclass selection. Upon activation, a naïve B cell can functionally tune its Ab response from IgM to the production of IgG, IgE, or IgA through class switch recombination (*29*), a highly regulated process largely controlled by cytokines and T helper–provided signals (*30*). IgG3 Ab responses are induced robustly during acute HIV infection but decline rapidly with progressive disease (*31*). Because IgG3 responses were induced by protein immunization alone (VAX003 at visit 5), it is unlikely that the viral vector prime alone was responsible for the selection of functional Ab responses. However, it is possible that the viral vector prime may have provided key adjuvanting signals preventing further class switch recombination to less functional subclasses during RV144 boosting and that protein immunization in alum alone, in the absence of additional strong inflammatory priming signals in VAX003, may have progressively driven class switch recombination from IgG3 to

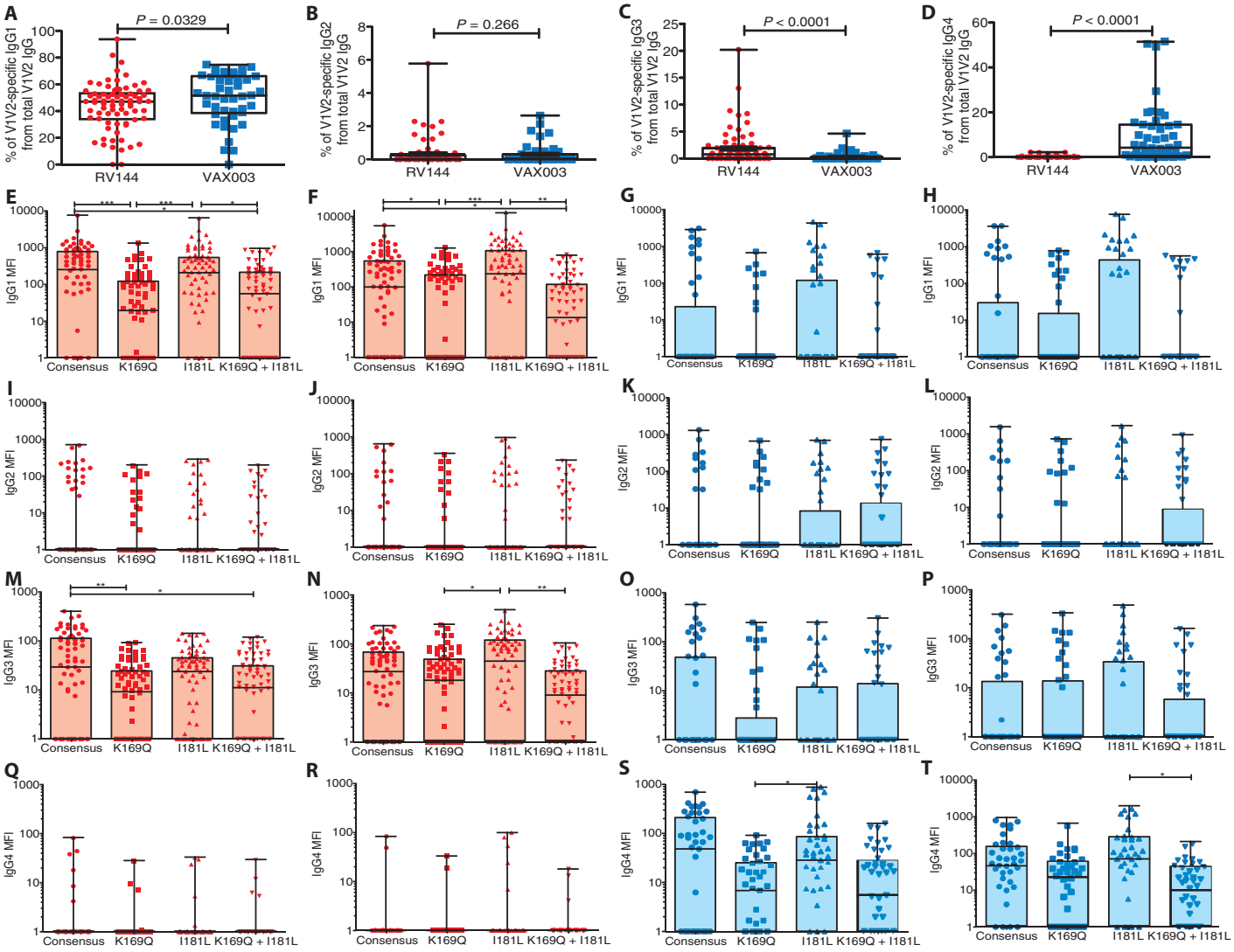

Fig. 6. gp70V1V2-specific IgG subclass profiles. (**A** to **D**) A customized Luminex subclass analysis of vaccine-induced gp70V1V2-specific Abs was performed on purified IgG and shows (A) IgG1, (B) IgG2, (C) IgG3, and (D) IgG4 Ab levels. Similarly, a customized Luminex subclass analysis was used to measure Ab subclass levels against V2 consensus and breakthrough sequences between consensus clade E (E, I, M, Q, G, K, O, and S) and vaccine-specific 92TH023_VCP1521/CM244 sequences (F, J, N, R, H, L, P, and T). V2 sequences including consensus, K169Q, I181L, and K169Q+ I181L sequences were compared. (**E** to **T**) IgG1 (E to H), IgG2 (I to L), IgG3 (third row, M to P), and IgG4 subclass levels (Q to T) are shown. RV144 samples are shown by red symbols, and VAX003 samples are shown by blue symbols. *P* values were determined by Kruskal-Wallis tests with Dunn's post hoc analyses. ***$P < 0.0001$, **$P < 0.01$, *$P < 0.05$.

the less functional IgG1 and finally to IgG4. Alternatively, IgG3 responses may be intrinsically transient because of their short serum half-life and may inevitably be lost in all vaccinees over time, as was observed in most RV144 vaccinees at week 52 (fig. S5). However, tetanus boosting specifically promotes IgG3 recall responses (*32*, *33*), suggesting that IgG3 responses are not inherently transient, are retained in memory, and can be efficiently recalled. Thus, future vaccine design efforts aimed at defining the signals that may program and boost IgG3 responses specifically in the absence of class switch recombination may promote greater polyfunctional protection from infection.

VAX003 samples showed higher gp120-specific IgG3 titers after the second gp120 vaccine compared to RV144; however, these titers decreased after the third VAX003 vaccination. These higher IgG3 levels did not coincide with elevated ADCC activity in VAX003 visit 5 samples. By contrast, RV144 vaccination induced levels of IgG3 similar to those observed in VAX003 at visit 7; yet, RV144 vaccinees exhibited significantly higher ADCC activity. Therefore, it is possible that, in addition to differences in IgG3 subclass selection, elevated IgG2 Abs induced by VAX003 at early time points may have competed and blocked the recruitment of ADCC activity, consistent with previous reports (*27*). Alternatively, the RV144 prime and boost may have induced IgG3 Abs with superior functionality, potentially via additional modifications to IgG3 Abs, such as modifications to the Ab glycan, as has been recently observed in HIV clinical samples (*34*). Thus, future glycan analysis of antigen-specific IgG3 Abs may provide key insights into the biophysical features of the most potent antiviral Ab responses.

The depletion of IgG3 Abs from RV144 samples highlights the functional importance of HIV-specific IgG3 responses. Specifically, IgG3 depletion resulted in decreases in Fc-mediated effector activities and the loss of polyfunctionality, suggesting that despite being low in abundance, IgG3 Abs are potent effectors of the vaccine-induced humoral immune response. Despite the negative correlation between IgG3 responses and functionality in VAX003 (Fig. 3), depletion of IgG3 also resulted in a loss of ADCP, but not ADCC, further emphasizing the functional importance of this low-abundance Ab subclass in driving Ab functionality. The unexpected lack of a correlation between total IgG3 levels and ADCP activity in VAX003 (Fig. 3) may be related to the strong association between IgG3 levels and elevated levels of less functional Ab subclasses (IgG2 and IgG4; fig. S4) that may compete within the bulk polyclonal pool to diminish functionality, rather than to a direct antagonistic effect of IgG3 Abs in VAX003. Because the induction of ADCC and ADCP requires the engagement of low-affinity Fc receptors, Abs must form immune complexes to recruit the effector function of the innate immune cells. Therefore, IgG3 Abs, which represent less than 10% of total circulating Abs, likely cooperate with IgG1 to drive Ab functionality. Along these lines, although IgG3 depletion eliminated the polyfunctional profile of RV144 Abs, it did not completely abrogate all Ab functionalities, suggesting that additional Ab subclasses, especially IgG1, may coordinately drive Ab functionality. Thus, future efforts aimed at defining additional functional IgG1 Ab subpopulations that may function independently or in synchrony with IgG3 may provide critical insights in the design of a vaccine aimed at eliciting effective control of HIV.

Because inflammatory cues are centrally linked to Ab subclass selection (*35*), it is possible that subclass selection differences among the trials could be driven by differences in the risk status of vaccinated populations. Specifically, RV144 was performed in low-risk individuals, and VAX003 was performed in higher-risk intravenous drug users, the latter potentially associated with elevated baseline immune activation. No differences were observed in baseline subclass selection. However, at baseline, RV144 samples bound to Fc receptors less robustly than Abs from VAX003 placebo samples (fig. S7), suggesting that immune activation, among high-risk drug users in the VAX003 population, may result in global secretion of Abs with an altered capacity to recruit Fc receptor activity. However, longitudinal follow-up of VAX003 vaccinees demonstrates that these subjects were able to elicit IgG3 Abs after the second vaccine and only skewed their Ab subclass selection to IgG4 Abs in the setting of seven vaccine boosts, arguing that differences in the vaccine regimen, rather than patient population differences, may account for differences in observed Ab effector profiles.

Whereas gp70V1/V2 Ab subclass skewing followed bulk gp120 Ab profiles in both trials, epitope mapping of IgG subclass–specific responses against the V2 crown shows that the AIDSVAX B/E V2 crown was immunogenic in both VAX003 and RV144. However, this region only induced IgG4 Abs in VAX003, likely due to repetitive boosting driving subclass switching to IgG4. By contrast, RV144 elicited IgG1/IgG3 Abs targeting the V2 crown; however, this coverage was limited, with reduced coverage of the K169Q breakthrough variant, potentially only conferring strain-specific immunity. Moreover, although the K169Q mutation appeared to dominate the I181X effect, differences in Ab recognition were observed in the presence of the I181X variant in the 92TH023 backbone, suggesting that certain residues at site 181 may conformationally affect Ab epitope exposure. The importance of the V1V2-K169Q mutation was confirmed in a complementary paper by Yates *et al.*, in which gp70 BCaseA2 V1V2 169K–specific IgG3 response rates predicted vaccine efficacy among RV144 vaccinees (*36*). However, whereas Yates *et al.* detected vaccine-induced IgG titers to gp120A244 protein in most RV144 vaccinees half a year after vaccination (week 52), in our studies, only 25% of subjects had detectable gp120MN-specific IgG responses at the same time point (fig. S8), likely due to the selection of different gp120 immunogens expressing different epitopes used to measure vaccine-induced Ab levels.

The studies performed here focused on humoral immune effector functions against gp120MN because MN responses were highly correlated to A244 responses within our preliminary Fc-effector function assays (fig. S2). However, it is critical to note that most of the breakthrough infections in the RV144 trial were clade E infections, suggesting that a caveat of this study was the predominant focus on the clade B immunogen. Thus, closer attention to clade E gp120A244 immune responses could offer critical insights into humoral immune profiles that may be associated with reducing risk of infection. However, Yates *et al.* observed differences in IgG1 reactivity against MN and A244, but more marginal differences in IgG3 responses against the different clade immunogens, suggesting that the data presented here related to IgG3 polyfunctionality may be less clade-dependent (*36*). Additionally, case controls were not available at the time of analysis; however, Yates *et al.* observed that vaccine-induced IgG3 correlated with reduced risk of infection in RV144. Future studies are required to determine whether polyfunctional IgG3 Fc-effector functions are present in response to gp120A244 protein and, more importantly, are correlated to reduced risk of infection within the case-control study samples.

In summary, both trials induced suboptimal Ab responses, but the RV144 prime/boost induced qualitatively superior polyfunctional V2-specific Ab responses compared with VAX003. These qualitative functional differences were marked by distinct IgG subclass selection profiles. Although IgG1/IgG3 Abs were induced in both trials, it is likely that differences in the protective efficacy observed may be attributable to differences in both the total level and relative prevalence of each IgG subclass induced against the neutralization-sensitive V2 loop or other Ab specificities. Thus, these data argue that vaccine strategies aimed at eliciting favorable subclass selection with broader coverage of the V2 loop may provide enhanced protection from infection. Moreover, measures of vaccine protective efficacy that move beyond titer alone and include variables such as Ab subclass and functional activity may provide critical new insights into the potential antiviral activity of Abs that extends beyond virus neutralization.

MATERIALS AND METHODS

Study design

This study was designed to test the hypothesis that the moderately protective phase 2B RV144 vaccine trial induced HIV-specific IgG with enhanced Fc-effector ability compared to the unprotective phase 3 VAX003 vaccine trial. Both vaccine trials included the same AIDSVAX B/E rgp120 protein vaccination within their vaccine regimens (RV144: two vaccinations, VAX003: seven vaccinations). IgG was purified from plasma samples taken from vaccinees/placebos of both trials at a time point 2 weeks after final vaccination. Purified IgGs were tested for multiple in vitro Fc-effector function assays, including gp120-specific ADCP, ADCC, and Ab-mediated NK activation. The IgG subclass profiles, as well as their HIV antigen specificities, were determined by Luminex.

IgG3 and IgG4 subclass Abs were depleted from a selection of samples, and Fc-effector assays were conducted to determine the relative contribution of the respective isotypes toward activation/inhibition of Fc-effector function. To further determine whether the isotype differences observed between the two vaccines were influenced by the higher number of AIDSVAX B/E protein boosts administered within the VAX003, plasma samples from time points 2 weeks after the second and after the third AIDSVAX B/E VAX003 vaccination were purified for IgG and characterized for Fc-effector responses and isotype profiles. Investigators were blinded to which plasma samples were placebo or vaccines.

Vaccine-induced Abs

RV144 (*1*) plasma samples included 100 participants, consisting of 20 placebo and 80 vaccinated subjects at weeks 0 and 26 (6.5 months), 2 weeks after the final vaccination (consisting of two ALVAC-HIV vCP1521 prime and two recombinant gp120 AIDSVAX B/E boosts, administered with ALVAC-HIV vCP1521), provided by the U.S. Military HIV Research Program (MHRP) and the RV144 study group. VAX003 (*2*) samples were obtained from 60 participants, consisting of 10 placebo and 50 vaccinated subjects at 30.5 months, 2 weeks after the final vaccination (visit 15: seven recombinant gp120 administrations), provided by Global Solutions for Infectious Diseases (GSID). VAX003 plasma samples from 50 vaccine recipients were also obtained at 1.5 months, 2 weeks after the second rgp120 vaccination (visit 5), and at 6.5 months, 2 weeks after the third rgp120 vaccination (visit 7), provided by GSID.

Bulk IgG purification

IgG was purified from all vaccine plasma and serum samples using Melon Gel columns according to the manufacturer's instructions (Thermo Scientific). Briefly, the resin was incubated with diluted plasma to allow for the binding of plasma proteins. Abs were spun through the resin, and the concentration was calculated using a NanoDrop spectrophotometer (Thermo Scientific).

gp120-binding titers

Enzyme-linked immunosorbent assay (ELISA) plates (Nunc) were coated overnight at 4°C with 80 μl of PBS containing recombinant gp120(MN) (250 ng/ml) (Immune Technology) per well; identical plates were blocked with 100 μl of PBSA [phosphate-buffered saline (PBS) containing 5% bovine serum albumin (BSA)] per well and used as sample background controls. The wells were washed six times with PBST (PBS containing 0.05% Tween 20), blocked with 100 μl of PBSA per well for 2 hours, and washed again. Serial dilutions of purified IgG, starting at 1 mg/ml, were then added to each well (81 μl) in replicate, and the plates were incubated at room temperature for 2 hours. A pool of Ig from HIV-positive individuals (HIV-IG) was used as a positive control. After six washes with PBST, 100 μl per well of a horseradish peroxidase–conjugated anti-human IgG (diluted 1:500 in PBS; R&D Systems) was added to the wells. The plates were incubated for 1 hour at room temperature, washed six times, and developed via the addition of 50 μl of *o*-phenylenediamine (0.4 mg/ml) in PBS/H_2O_2. The reaction was stopped by the addition of 50 μl per well of stop solution (2.5 M H_2SO_4), and the optical density at 492 and 605 nm was read on a Tecan ELISA reader.

THP-1 phagocytosis assay

The THP-1 phagocytosis assay was performed as previously described (*37*). Briefly, THP-1 cells (American Type Culture Collection) were cultured as recommended. Biotinylated rgp120(MN) (Immune Technology) was used to saturate the binding sites on 1-μm fluorescent neutravidin beads (Invitrogen) overnight at 4°C. Excess antigen was removed by washing the pelleted beads, which were then incubated with patient Ab samples for 2 hours at 37°C. After opsonization, THP-1 cells were added to the bead/Ab mix and incubated overnight to allow for phagocytosis. Cells were then fixed, and bead uptake was measured using flow cytometry on a BD LSRII equipped with high-throughput sampler. Phagocytic scores are presented as the integrated MFI (iMFI; frequency × MFI) (*38*). Each Ab sample was tested over a range of concentrations (0.1 to 100 μg/ml).

Rapid fluorometric ADCC assay

A modified rapid fluorometric ADCC (RFADCC) assay was used (*39*, *40*). In brief, the CEM-NKr $CCR5^+$ T lymphoblast cell line was pulsed with rgp120(MN) (60 μg/ml) and then labeled with the intracellular dye carboxyfluorescein diacetate succinimidyl ester (CFSE) and the membrane dye PKH26. NK cells were isolated from healthy donor whole blood with RosetteSep (STEMCELL Technologies). Purified IgG was added to the labeled CEM-NKr cells, which were then incubated with NK cells for 4 hours at 37°C at a 1:5 ratio target to effector cells. The cells were fixed, and the proportion of $PKH26^+$ cells that had lost intracellular CFSE staining (that is, lysed target cells) was determined using flow cytometry. All samples were tested in duplicate at the final concentration of 100 μg/ml.

NK degranulation and intracellular cytokine staining

An assay to determine the expression of surface CD107a and intracellular production of IFN-γ and MIP-1β was performed by pulsing the CEM-NKr $CCR5^+$ T lymphoblast cell line with rgp120(MN) (30 μg/ml). NK cells were isolated from whole blood from healthy donors using negative selection with RosetteSep (STEMCELL Technologies), as recommended by the manufacturer. The CEM-NKr cells and isolated primary NK cells were mixed at a ratio of 1:5, and purified IgG, anti–CD107a–phycoerythrin (PE)–Cy5 (BD), brefeldin A (10 μg/ml) (Sigma), and GolgiStop (BD) were added for 5 hours at 37°C. The cells were then first stained for surface markers using anti–CD16–allophycocyanin (APC)–Cy7 (BD), anti–CD56-PE-Cy7 (BD), and anti–CD3–Alexa Fluor 700 (BD) and then stained intracellularly with anti–IFN-γ–APC (BD) and anti–MIP-1β–PE (BD) using Fix and Perm A and B solutions (Invitrogen). The cells were then fixed in 4% paraformaldehyde and analyzed using flow cytometry. NK cells were defined as CD3-negative and CD16- and/or CD56-positive.

Customized Luminex subclass assay

A customized Luminex subclass assay (*41*) was used to quantify the relative concentration of each Ab isotype among the HIV antigen-specific Abs. Briefly, carboxylated microspheres (Luminex) were coupled with rgp120(MN) protein, rgp140(MN) protein, V1/V2 scaffold (provided by L. Liao, Duke University), or V2 breakthrough peptides (*9*) [consensus clade E: RNCSFNMTTELRDKKQKVHALFYKLDIVQI; K169Q: RNCSFNMTTELRDKQQKVHALFYKLDIVQI; I181L: RNCSFNMTTELRDKKQKVHALFYKLDLVQI; K169Q + I181L: RNCSFNMTTELRDKQQKVHALFYKLDLVQI, and vaccine-specific 92TH023_VCP1521/CM244 consensus (identical in sequence): RNCSFNMTTELRDKKQKVHALFYKLDIVPI; 92TH023_VCP1521_K169Q: RNCSFNMTTELRDKQQKVHALFYKLDIVPI;92TH023_VCP1521_I181L: RNCSFNMTTELRDKKQKVHALFYKLDLVPI; 92TH023_VCP1521_K169Q

+I181L: RNCSFNMTTELRDKQQKVHALFYKLDLVPI] by covalent NHS-ester linkages by combining EDC and NHS (Thermo Scientific) in PBS per the manufacturer's instructions. The loaded microspheres (50 µl of 100 microspheres/µl in 0.1% BSA/PBS) were added to each well of a 96-well filter plate (Millipore). Each vaccine sample (50 µl of purified bulk IgG diluted to 100 µg/ml) was added to five replicate wells of the 96-well plate and incubated overnight at 4°C. The microspheres were then washed three times with 100 µl of PBST. IgG detection reagents specific for IgG1, IgG2, IgG3, IgG4, or pan-IgG conjugated with PE (Southern Biotech) were added individually to the replicate wells containing the bound vaccine-induced Abs. The 96-well plate was incubated with shaking for 2 hours, washed three times, and read on a Bio-Plex 200 System. The background signal, defined as the MFI of microspheres incubated with detection reagents in the absence of clinical Abs, was subtracted from each sample.

IgG3 and IgG4 depletion

IgG3 and IgG4 capture beads were made by reacting 10 mg of M-270 Streptavidin Dynabeads (Invitrogen) with 100 µg of a biotin-conjugated IgG3- or IgG4-specific Ab (Southern Biotech). The beads were washed three times with 500 µl of PBS and then incubated with the Ab (in PBS) for 30 min at room temperature with gentle rotation. After conjugation, the beads were washed three times with 0.1% BSA in PBS and stored at a final concentration of 10 mg/ml. For IgG3 and IgG4 depletion, 60 µl of patient IgG at 1 mg/ml was added to 50 µl of the respective beads and incubated at 4°C for 24 hours. After 24 hours, the beads were removed via magnetic separation, and the supernatant was collected. A Luminex-based subclassing assay was run before and after the bead incubation to assess efficient IgG3/IgG4 depletion. If depletion was not sufficient, the above method was repeated again. For IgG3 Abs, after depletion, there was an 86% decrease in the HIV-specific IgG3 signal in the RV144 samples (pre-depletion ratio IgG3/bulk IgG: 0.0663, post-depletion ratio: 0.00918, $P = 0.0006$). However, because of the low initial titer of IgG3 Abs present in the VAX003 samples, a small but significant reduction in the IgG3 signal was achieved (pre-depletion ratio IgG3/bulk IgG: 0.00508, post-depletion ratio: 0.00377, $P = 0.0005$). For IgG4 Abs, a 91% ($P = 0.0005$) decrease in HIV-specific IgG4 was achieved for VAX003 samples, whereas because of the almost nonexistent IgG4 Ab population present in RV144 samples, no significant decrease in IgG4 titers was achieved. A selection of the IgG3/IgG4 samples, before and after depletion, was also assessed for their remaining non-depleted IgG subclass profiles. No nonspecific IgG subclass loss was observed.

Statistical analysis

Statistical analysis was performed with GraphPad Prism and SAS 9.2. Mann-Whitney test was used to compare continuous study outcomes between the two trials. Rank-based analysis of covariance (ANCOVA) was used to adjust for the effect of gp120-specific Ab titers while comparing the continuous study outcomes between the two trials. For paired comparisons of continuous study outcomes, Wilcoxon signed-rank tests were used, whereas for analysis of continuous study outcomes consisting of three or more groups, Kruskal-Wallis tests with Dunn's post hoc analyses were used. Fisher's exact test was used to compare categorical study outcomes between the two trials. Spearman rank correlations were used to examine bivariate associations between continuous study outcomes. All P values are two-sided, and a P value of less than 0.05 was considered significant.

SUPPLEMENTARY MATERIALS

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Fig. S1. Differences between RV144 and VAX003 Fc-mediated Ab functions.
Fig. S2. Fc-effector functions correlate between gp120MN and gp120A244 for both vaccine trials.
Fig. S3. Ab Fc-function correlation differences between RV144 and VAX003.
Fig. S4. Antigen-specific IgG subclass correlations for RV144 and VAX003 samples.
Fig. S5. Correlation of Fc-effector function from bulk and IgG3- and IgG4-depleted RV144 and VAX003 samples.
Fig. S6. Evolving relationship between VAX003 gp120-specific IgG subclass and ADCP.
Fig. S7. Differential binding of RV144 and VAX003 bulk purified IgG samples to Fcγ receptors.
Fig. S8. gp120MN-specific IgG binding over time.

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