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# Internalization of HIV-1 by Phagocytes Is Increased When Virions Are Opsonized with Multimeric Antibody in the Presence of Complement

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**ABSTRACT** The low abundance of envelope spikes and the inability of IgG to aggregate virions render HIV-1 an inadequate target for antibody-mediated clearance by phagocytes. In an attempt to improve the ability of antibody to mediate the internalization of HIV-1 virions, we generated multimers of the broadly neutralizing HIV-1-specific monoclonal antibody (MAb) VRC01 using site-directed mutagenesis of the Fc segment. We then measured virion internalization using primary human monocytes and neutrophils. We found that, in the absence of complement, immune complexes consisting of HIV-1 virions and VRC01 multimers were slightly more efficiently internalized than were complexes formed with monomeric VRC01. The presence of complement, however, greatly augmented internalization of immune complexes formed with the multimeric MAb but had little impact on monomeric MAb-mediated internalization. Multimerization and the presence of complement overcome the limited ability of monomeric antibody to mediate internalization of HIV-1 virions and may thus provide a therapeutic approach to clearing virus.

**IMPORTANCE** Antibody-mediated internalization of HIV-1 by phagocytes, a potential mechanism for clearing virus, is very inefficient. In an effort to improve viral clearance, we produced a multimeric form of the broadly neutralizing monoclonal antibody VRC01. We found that VRC01 antibody multimers (primarily hexamers) were only slightly more efficient in mediating HIV-1 internalization than was monomeric VRC01. However, the addition of complement resulted in substantially greater internalization of multimer-opsonized virus. In contrast, complement had little if any impact on internalization of monomer-opsonized virus. Therefore, antibody multimerization in combination with complement may overcome the limited ability of monomeric antibody to mediate internalization of HIV-1 virions. Our findings may provide a therapeutic approach to clearing virus.

**KEYWORDS** antibody, complement, Fc receptors, HIV-1, phagocytosis

Antibodies play a pivotal role in preventing and controlling human immunodeficiency virus 1 (HIV-1) infection (1). Besides neutralization, antibodies mediate various effector functions through their Fc domain by interacting with Fcγ receptors (FcγRs) or with complement (2). Examples include antibody-dependent cellular cytotoxicity and antibody-dependent cellular phagocytosis (2). Both effector functions have been studied in different experimental settings, including nonhuman primate models and a human HIV-1 efficacy trial (RV144) (3–6).

In the setting of viral infections, antibody-dependent internalization occurs when antibodies opsonizing an infected cell or a virion cross-link FcγRs or complement receptors on phagocytes such as monocytes, macrophages, and neutrophils. Such cross-linking results in the uptake and, generally, the inactivation of the virions or infected cells within the phagolysosome. For several viruses, antibody-dependent internalization is thought to play

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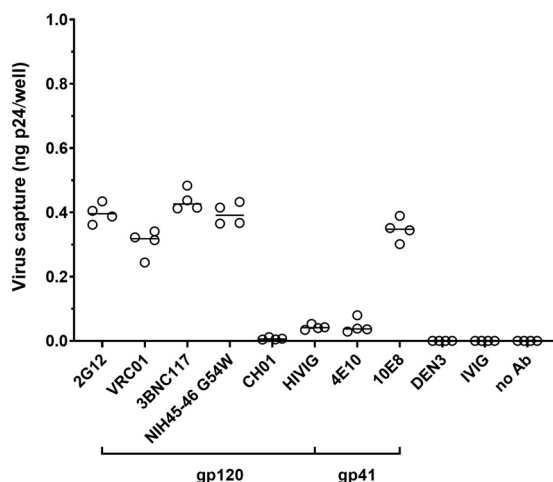
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**FIG 1** Envelope-specific antibodies differentially capture HIV-1<sub>IGFP/JRFL</sub> virions. Virus capture was measured by determining the total p24 content of captured HIV-1<sub>IGFP/JRFL</sub> virions by p24 ELISA. All envelope-specific antibodies (gp120-specific and gp41-specific) as well as controls were tested at 2  $\mu$ g/mL. Experiments were performed in triplicate and repeated four times. Lines represent median p24 capture. HIVIG is IgG from pooled HIV-positive donors; IVIG (intravenous immunoglobulin) is IgG from pooled HIV-negative donors.

a major role *in vivo* in clearing virus (7). However, a beneficial *in vivo* role has not been confirmed with respect to preventing or controlling HIV-1 infection (8).

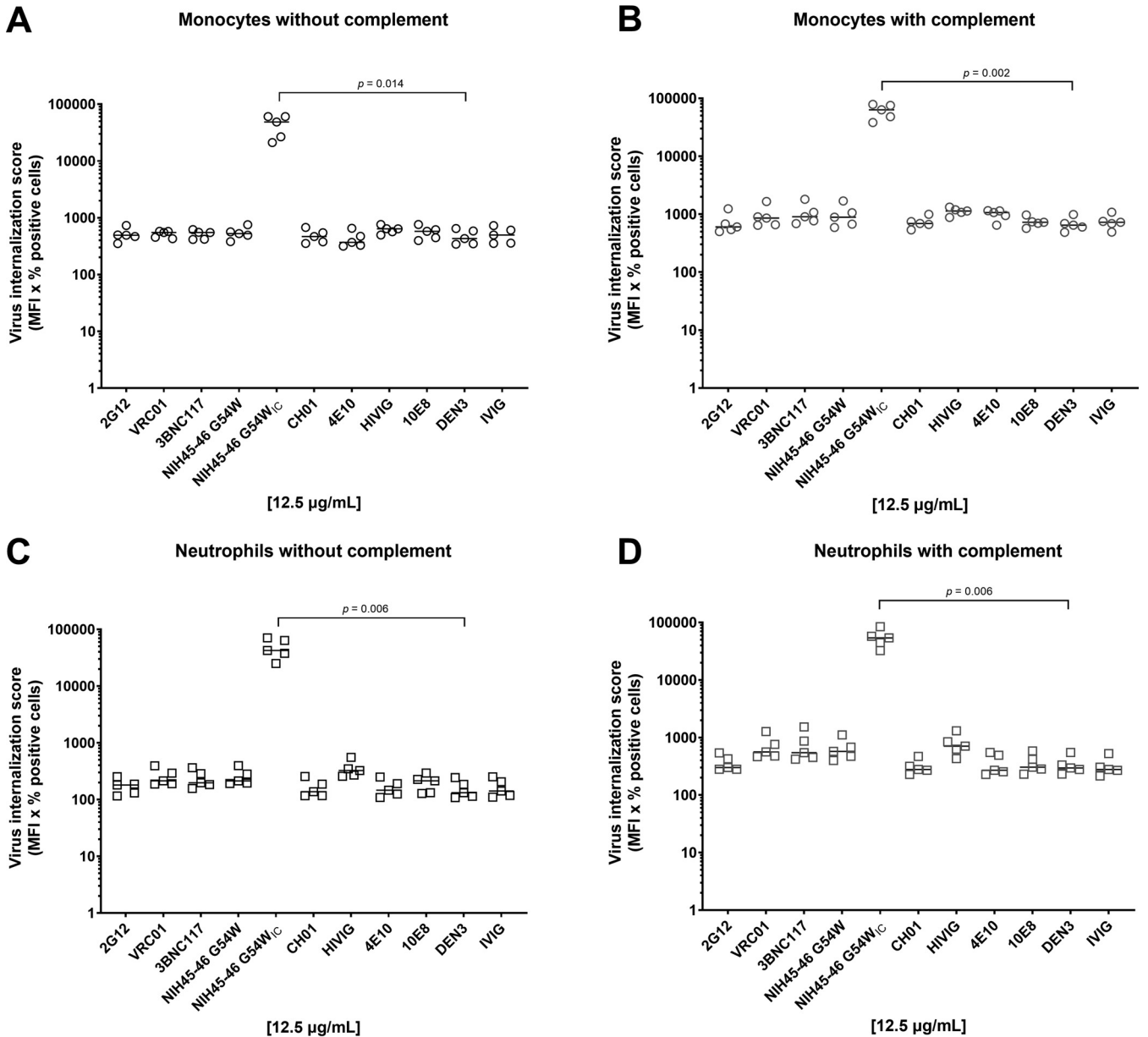
As reported earlier by our group, immune complexes made from HIV-1 virions and HIV-1-specific antibodies are inadequate targets for phagocytosis (9). Limiting factors such as particle size and the paucity of envelope spikes on the virion surface underlie the inefficient uptake of opsonized HIV-1 virions. We had also found that experimentally aggregating virus or increasing the abundance of HIV-1 envelope spikes substantially enhanced the internalization of HIV-1 particles opsonized with antibody (9). However, these *in vitro* manipulations required to improve internalization cannot be accomplished *in vivo*.

In this study, we sought to improve the therapeutic potential of antibodies by producing multimers of the broadly neutralizing monoclonal antibody (MAb) VRC01 and testing the ability of the multimers to mediate internalization of HIV-1 virions.

## RESULTS

**Anti-HIV-1-specific antibodies capture virus but mediate limited internalization by monocytes or neutrophils.** We first assessed the ability of a panel of HIV-1-specific antibodies to capture HIV-1<sub>IGFP/JRFL</sub> virions using a p24 readout as a measure of total virus captured. As reported previously (9), we found distinct capture profiles of the HIV-1 gp120- and gp41-specific antibodies (Fig. 1). As expected, the HIV-1-specific MAb CH01 was not able to capture HIV-1<sub>IGFP/JRFL</sub> virions, consistent with its previously reported inability to neutralize HIV-1<sub>JRFL</sub> (10). The negative-control antibodies DEN3 (anti-dengue NS1 human IgG1 MAb) and intravenous immunoglobulin (IVIG) did not capture HIV-1<sub>IGFP/JRFL</sub> virions.

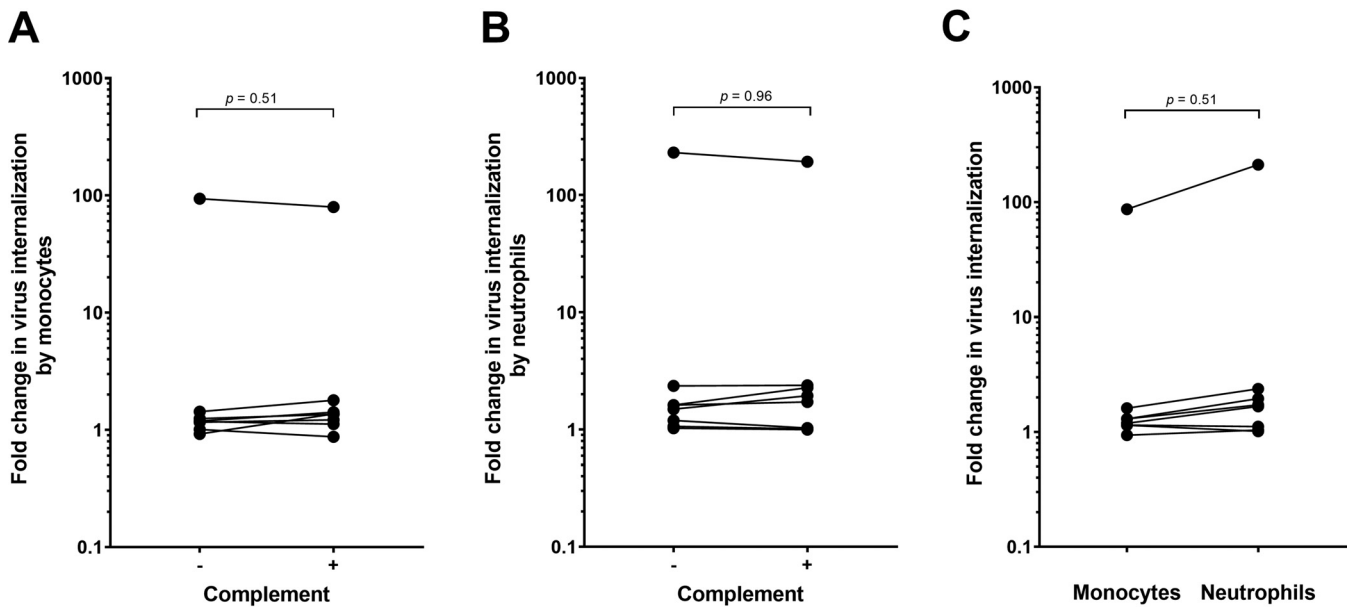
We next investigated whether virus captured by antibody was internalized by peripheral blood monocytes or neutrophils in either the presence or absence of complement. Neutrophils were incubated with diphenyleneiodonium chloride (DPI) prior to incubation with antibody-virus complexes to block neutrophil extracellular trap (NET) formation. The latter mechanism has been shown to actively capture and eliminate HIV-1 virions *in vitro* (11), thus potentially interfering with our internalization assay. As previously reported by our group, we observed no significant antibody-mediated internalization of virions ( $P > 0.05$  for all HIV-specific MAbs compared with the DEN3 control or for HIVIG compared with IVIG; Kruskal-Wallis with Dunn's correction) by monocytes (Fig. 2A) (9) or by neutrophils (Fig. 2C). Note, however, that immune complexes formed by cross-linking antibody-opsonized virions with a goat anti-human antibody (NIH45-46 G54W<sub>C</sub>) resulted in >80-fold internalization compared with the DEN3



**FIG 2** Antibody opsonized HIV-1<sub>GFP/JRFL</sub> virions are inefficient targets for primary phagocytes. (A to D) Uptake of HIV-1<sub>GFP/JRFL</sub> virions by monocytes (A) or neutrophils (C) in the absence of complement or by monocytes (B) or neutrophils (D) in the presence of complement was measured using MAbs at a final concentration of 12.5 µg/mL and the polyclonal antibodies HIVIG and IVIG at a final concentration of 50 µg/mL. NIH45-46 G54W<sub>c</sub> was included as a positive control. All experiments were performed in triplicate and were repeated five times with different healthy donors. Solid lines indicate median virus internalization.

control ( $P < 0.05$ ; Kruskal-Wallis with Dunn’s correction), thus confirming that cellular internalization function was intact (Fig. 2A to D). Addition of normal guinea pig serum as a source of complement did not improve monomer-mediated internalization of virions in monocytes (Fig. 2B and Fig. 3A) or neutrophils (Fig. 2D, Fig. 3B). In addition, overall antibody-mediated internalization by the monomeric MAbs, when expressed as a fold change compared to the negative-control MAb, did not differ between monocytes and neutrophils (Fig. 3C).

**Multimerization of VRC01.** We had previously demonstrated that poor antibody-mediated internalization of HIV-1 was due to the scarcity of Env spikes on HIV-1 virions and the inability of IgG antibody to aggregate virions (9). In an attempt to overcome these factors, we generated multimers of VRC01 by mutating key amino acids in Fc. Using site-directed mutagenesis, we changed three amino acids (E345R, E430G, and



**FIG 3** (A and B) Monomeric MAb-mediated internalization of virions is similar with and without complement on monocytes (A) and on neutrophils (B). Each data point represents the median fold change of the internalization score for the seven antibodies (Abs) that capture HIV-1<sub>iGFP/JRFL</sub> (2G12, VRC01, 3BNC117, NIH46 G54W, HIVIG, 10E8, and 4E10) as well as for the immune complexes formed by cross-linking NIH45-46 G54W with a goat anti-human antibody (data points with the highest values; Fig. 1). When expressed as the median fold change (combining median values with and without complement), antibody-mediated internalization by monocytes is similar to internalization by neutrophils for the seven monomeric Abs, though there may be slightly better internalization of the anti-human antibody-cross-linked immune complex on neutrophils (C). Mann-Whitney tests were used to calculate the *P* values.

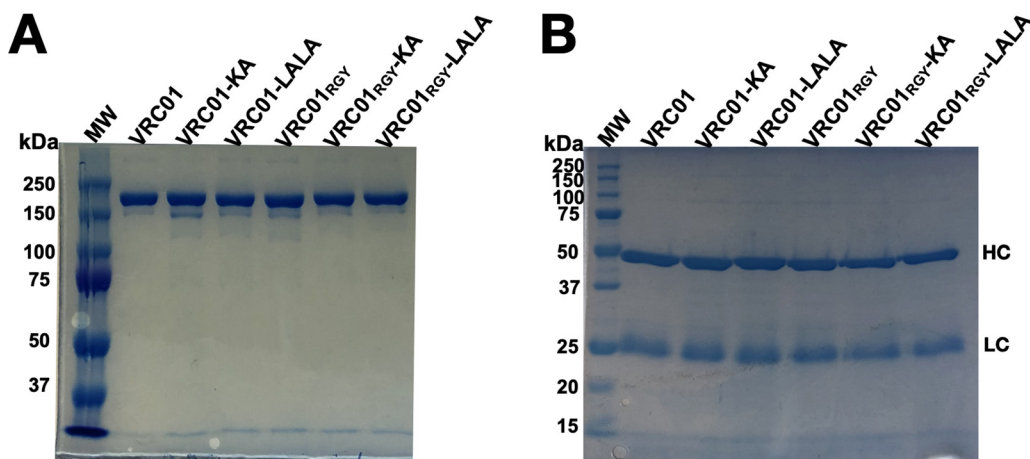
S440Y) known to result in IgG multimerization (12). We also introduced mutations to alter Fc $\gamma$ R and complement binding (LALA) or complement binding only (KA) (13). All VRC01 antibody variants were tested for integrity and purity under nonreducing and reducing conditions using SDS-PAGE. Under nonreducing conditions, we found that all antibody variants demonstrated a predominant band between 150 kDa and 250 kDa representing intact IgG (Fig. 4A). Addition of dithiothreitol as a reducing agent to the antibody variants resulted in bands representing the heavy-chain (50 kDa) and light-chain (25 kDa) fragments (Fig. 4B).

Size-exclusion chromatography (SEC) revealed that the multimeric antibodies VRC01<sub>RGY</sub>, VRC01<sub>RGY</sub>-LALA, and VRC01<sub>RGY</sub>-KA formed hexamers (1,030-kDa peaks) in solution (Fig. 5). Smaller peaks at 150 kDa indicated residual monomers. Both peaks were connected by a trace above the baseline level, suggesting a dynamic interconversion between the different oligomeric states (14). In contrast, the chromatogram of the parental monomeric VRC01 exhibited a predominant peak at 150 kDa (monomers) and a smaller peak around 412 kDa, indicating some degree of aggregation (Fig. 5).

Next, we analyzed the ability of the antibodies to bind recombinant HIV-1 gp140 in an enzyme-linked immunosorbent assay (ELISA) and to capture HIV-1<sub>iGFP/JRFL</sub> virions. As expected, antibody binding to gp140 was not affected by the Fc mutations resulting in VRC01 multimers or resulting in multimers and monomers with altered Fc $\gamma$ R and complement binding (Fig. 6A). We also found no significant difference between the median dissociation constant ( $K_d$ ) values of any of the VRC01 variants (Fig. 6B). The multimeric VRC01<sub>RGY</sub> variants were slightly better able to capture HIV-1 virions than were the monomeric VRC01 variants ( $P = 0.006$ , Kruskal-Wallis; Fig. 6C), although differences were nonsignificant when applying Dunn's multiple-comparison test.

In total, these results indicate that multimerization of VRC01 did not affect HIV-1 gp140 binding or virus capture, nor did the introduction of Fc-binding-site mutations or complement-binding site mutations.

**VRC01 multimerization enhances internalization of HIV-1 by primary monocytes and neutrophils.** To evaluate the ability of the engineered antibody variant VRC01<sub>RGY</sub> to mediated internalization, we carried out additional assays with primary monocytes



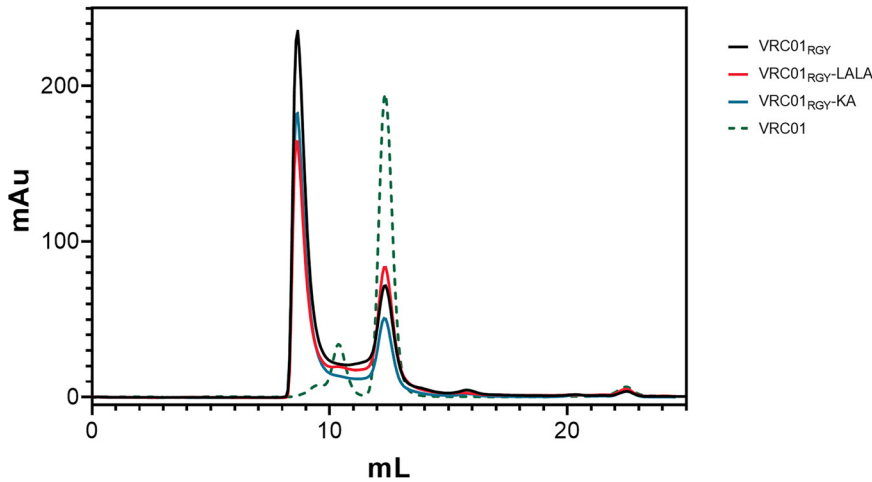
**FIG 4** Coomassie staining of purified VRC01 variants. (A and B) All VRC01 variants were examined with SDS-PAGE under nonreducing (A) and reducing (B) conditions. The percentage of acrylamide in the nonreducing gel (7.5%) differs from that in the reducing gel (10%). A total amount of 3  $\mu$ g of each antibody was loaded per well. Lane MW contains the All Blue Prestained Protein Standard (Bio-Rad). Heavy-chain (HC) and light-chain (LC) bands are indicated.

and neutrophils in the presence and absence of complement. We found that in the absence of complement, VRC01<sub>RGY</sub> and VRC01 mediated little internalization by monocytes compared to the negative-control antibody DEN3, although VRC01<sub>RGY</sub> was slightly, but not significantly, more effective than VRC01 (1.5-fold increase over DEN3 for VRC01<sub>RGY</sub> versus 1.1-fold increase over DEN3 for VRC01;  $P = 0.31$ ; Fig. 7A). Similarly, with neutrophils, VRC01<sub>RGY</sub> resulted in a 2.1-fold increase and VRC01, a 1.7-fold increase in internalization compared with DEN3 ( $P = 0.70$ ; Fig. 7B). The slightly increased internalization by the multimers may be due to their slightly increased ability to capture virions (Fig. 6C). In contrast, the immune complexes made by cross-linking monomeric VRC01-opsonized virions with a goat anti-human antibody (VRC01<sub>IC</sub>) induced a substantial uptake of virions in both cell types compared to the negative MAb ( $P = 0.002$ ; Fig. 8A).

Unlike the minimally increased virion uptake observed in the absence of complement, its addition substantially augmented VRC01<sub>RGY</sub>-mediated internalization by monocytes (8.0-fold increase compared to VRC01;  $P = 0.007$  and a 7.5-fold increase compared to DEN3 Fig. 7C) and by neutrophils (7.8-fold increase compared to VRC01;  $P = 0.12$  and a 19.4-fold increase compared to DEN3; Fig. 7D). Moreover, VRC01<sub>RGY</sub> mediated significantly greater internalization with complement than without on both monocytes ( $P = 0.002$ ; Fig. 7E) and neutrophils ( $P = 0.002$ ; Fig. 7F). For monomeric VRC01, the addition of complement resulted in no increased internalization compared to DEN3 by monocytes (Fig. 7C) and a 2.2-fold, nonsignificant increase by neutrophils (Fig. 7D). There was no statistically significant increase in internalization by VRC01 with complement compared to no complement on monocytes ( $P = 0.09$ ; Fig. 7E) or neutrophils ( $P = 0.09$ ; Fig. 7F). Internalization of virions by the VRC01<sub>IC</sub> control was little changed by the addition of complement with monocytes, whereas with neutrophils, we found a slight decrease in the presence of complement ( $P = 0.041$ ; Fig. 8B).

These results indicate that multimeric antibody can augment phagocyte-cell internalization of opsonized virions, but substantial increases in antibody-mediated internalization by multimeric antibody depend on the presence of complement.

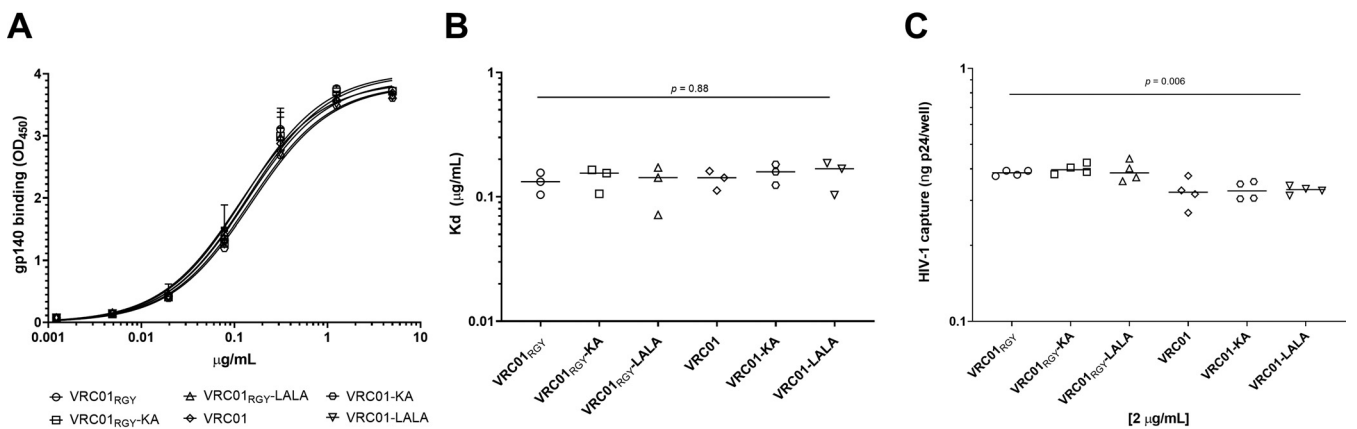
**Fc engagement with complement is critical for augmented antibody-mediated internalization by multimeric VRC01.** To decipher whether Fc $\gamma$ R- or complement-binding site mutations impact VRC01<sub>RGY</sub>-mediated internalization, we compared VRC01<sub>RGY</sub> with VRC01<sub>RGY</sub>-LALA and with VRC01<sub>RGY</sub>-KA. We found that both mutations resulted in lower uptake by monocytes and by neutrophils (Fig. 9A and B). Compared to VRC01<sub>RGY</sub>, VRC01<sub>RGY</sub>-KA reduced uptake 3.5-fold on monocytes ( $P = 0.007$ ) and 3.5-fold on neutrophils ( $P = 0.021$ ). VRC01<sub>RGY</sub>-LALA also resulted in less uptake than VRC01<sub>RGY</sub> on monocytes and



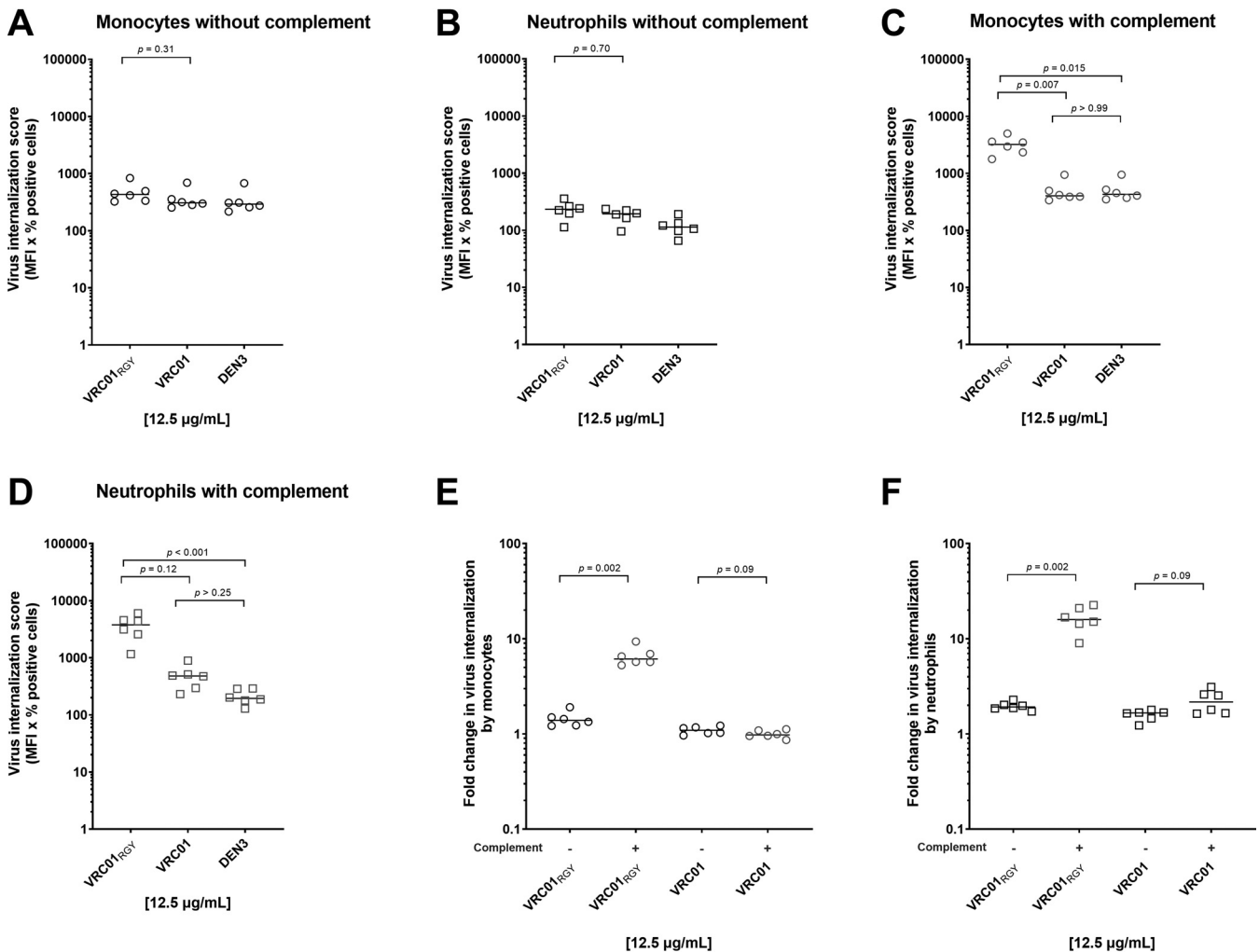
**FIG 5** E345R, E430G, and S440Y triple mutation promotes VRC01 multimerization. Size exclusion chromatography of purified multimeric VRC01 variants (VRC01<sub>RGY</sub>, VRC01<sub>RGY</sub>-LALA, and VRC01<sub>RGY</sub>-KA) was performed on a Superdex 200 increase 10 300 GL column. Absorbance at 214 nm was plotted against elution volume for each antibody. A protein standard with known molecular mass was loaded as a control to determine the size distribution of the peaks. Monomeric VRC01, obtained through the NIH AIDS Reagent Program, was included as a reference.

neutrophils, but the reductions were smaller (2.6-fold and 2.3-fold on monocytes and neutrophils, respectively), and the difference was only significant on monocytes ( $P = 0.039$ ). There was no significant difference between VRC01<sub>RGY</sub>-KA and VRC01<sub>RGY</sub>-LALA on either cell type (Fig. 9A and B). Since both the KA and LALA mutations affect complement binding, these findings suggest an important role of complement in the improved VRC01<sub>RGY</sub>-mediated internalization of HIV-1<sub>IGFP/JRFL</sub> but do not rule out a role of Fc-Fc $\gamma$ R engagement as well.

To investigate the impact of multimerization on binding of immune complexes to Fc $\gamma$ RIIA (a key activating Fc $\gamma$ R on both monocytes and neutrophils [15]), we developed an in-solution capture assay to measure the amount of HIV-1<sub>IGFP/JRFL</sub> bound by VRC01 variants in complex with soluble Fc $\gamma$ RIIA in the presence or absence of complement. Virus bound to VRC01 and VRC01-KA was similarly captured by Fc $\gamma$ RIIA, with or without complement, consistent with the fact that the KA mutation does not affect Fc $\gamma$ RIIA



**FIG 6** VRC01 Fc fragment mutations do not impact gp140 binding; however, VRC01 multimers capture slightly more virus than monomers. VRC01 multimers and monomers were tested for HIV-1<sub>IGFP/JRFL</sub> gp140 binding in an ELISA (A). Curve fitting was used to calculate  $K_d$  values of each antibody variant at half-maximum binding.  $K_d$  values of VRC01 variants did not differ ( $P = 0.88$ ; Kruskal-Wallis test) (B). All ELISA binding experiments were performed in triplicate and repeated three times. Lines represent median  $K_d$  values. All VRC01 variants were able to capture free HIV-1<sub>IGFP/JRFL</sub> virions (C). VRC01 multimers captured slightly more virus than monomers ( $P = 0.006$ ; Kruskal-Wallis test). However, when analyzed for differences between the antibody groups (Dunn's test), we found no significant difference between the antibodies. Virus capture was measured by p24 readout. All VRC01 variants were tested at 2  $\mu$ g/mL. Capture assays were carried out in triplicate and repeated four times. Solid lines represent median p24 capture.



**FIG 7** VRC01 multimerization augments HIV-1 internalization in the presence of complement. (A to D) VRC01<sub>RGY</sub><sup>-</sup> and VRC01-mediated uptake was measured in the absence of complement with monocytes (A) or neutrophils (B) and in the presence of complement with monocytes (C) or neutrophils (D). (E and F) VRC01<sub>RGY</sub>-mediated significantly greater HIV-1<sub>IGFP-JRF1</sub> internalization in the presence of complement compared to VRC01 in both monocytes (E) and neutrophils (F). Results are reported as virus internalization score (percent positive cells × MFI) or fold change compared to the DEN3 control. Experiments were performed in triplicate and were repeated with cells from the blood of six healthy donors. Solid lines indicate median internalization score or fold change in viral uptake. *P* values were calculated using Kruskal-Wallis with Dunn's multiple-comparisons tests (panels A to D) or using Mann-Whitney tests (panels E and F).

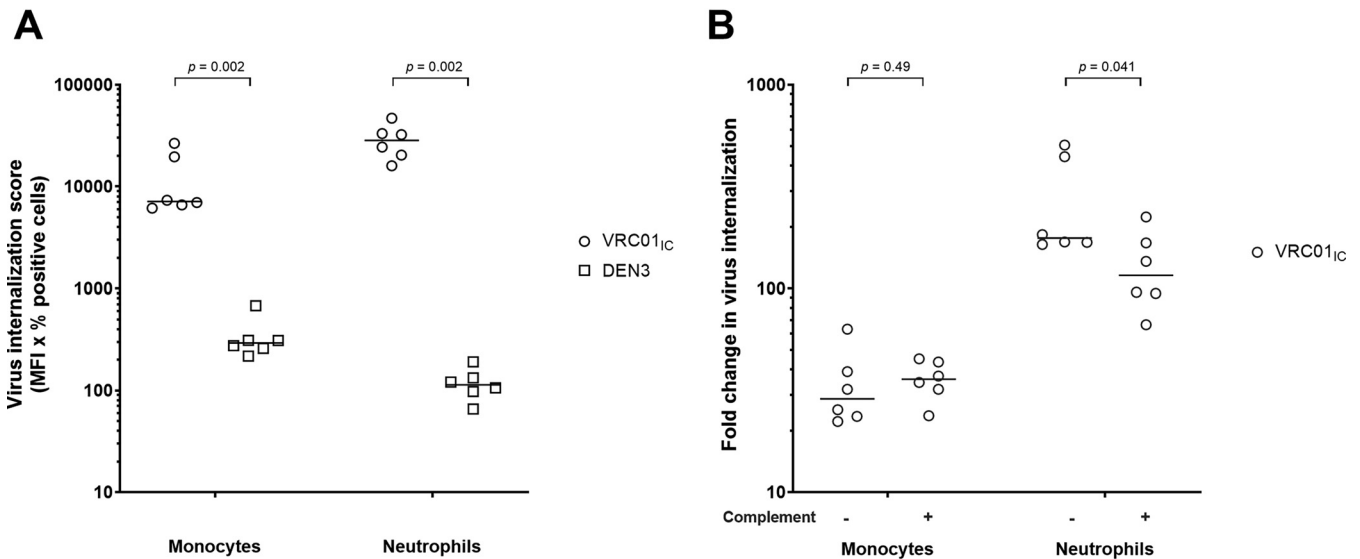
binding (13) (Fig. 10A and B). Also consistent with the known ability of the LALA mutation to greatly reduce Fc $\gamma$ RIIA binding, little if any virus bound to VRC01-LALA was captured by Fc $\gamma$ RIIA with or without complement (Fig. 10A and B).

In the absence of complement, multimerized VRC01 variants revealed slightly less virus capture than the monomers. Most strikingly, VRC01<sub>RGY</sub> did not interact with Fc $\gamma$ RIIA in the presence of complement (Fig. 10B). However, the KA mutation on VRC01<sub>RGY</sub> multimers restored much of the binding to Fc $\gamma$ RIIA, suggesting that complement shields the multimer's Fc regions from interaction with Fc $\gamma$ RIIA. This shielding effect was not observed with VRC01 monomers, presumably because monomeric IgG1 is not efficient at binding complement. As expected, both the monomeric and multimeric LALA mutants exhibited similarly poor interactions with Fc $\gamma$ RIIA.

## DISCUSSION

We previously reported that antibody-mediated internalization of HIV-1 by monocytes is ineffective *in vitro* (9). By aggregating virus or by increasing Env density on the surface of virions, more efficient internalization resulted (9). However, such manipulations of virions *in*

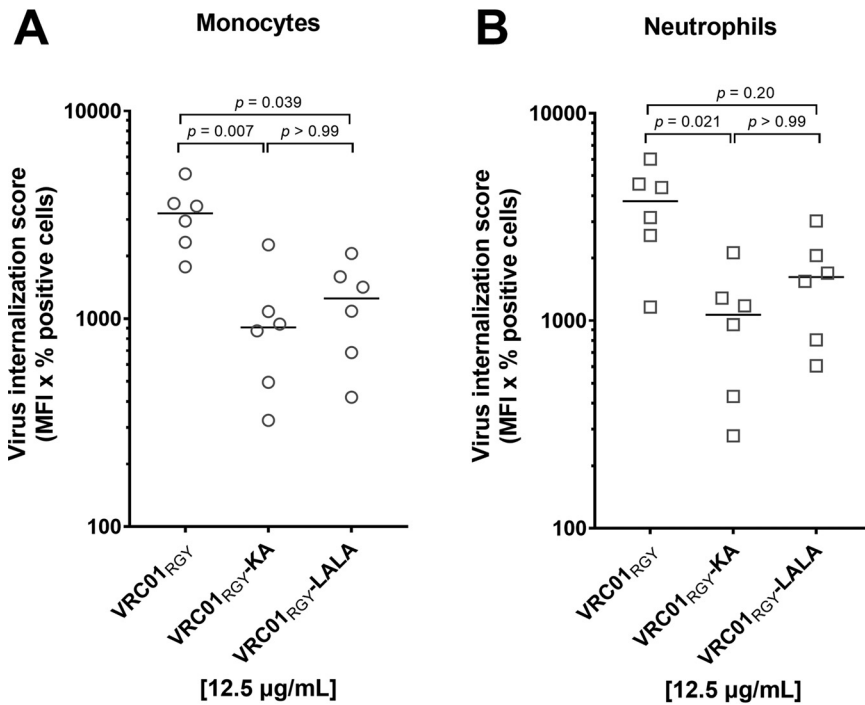




**FIG 8** Enhanced internalization of VRC01 opsonized virions cross-linked with anti-human antibody. Immune complexes made by cross-linking VRC01-opsonized virions with a goat anti-human antibody (VRC01<sub>1C</sub>) induced a substantial uptake of virions in both monocytes and neutrophils compared to DEN3 (A). Addition of complement slightly decreased ( $P = 0.041$ ) the uptake of HIV-1<sub>IGFP-JRFL</sub> virions by neutrophils but not by monocytes (B).  $P$  values were calculated using Mann-Whitney tests. Results are reported as virus internalization score (percent positive cells  $\times$  MFI) or fold change compared to the DEN3 control. Experiments were performed in triplicate and were repeated using cells from six healthy donors. Solid lines indicate medians.

*in vitro* would have little relevance in clinic settings. In the current investigation, we first determined if neutrophils might be better able to internalize opsonized virus. In addition, we evaluated the ability of a multimeric IgG1 to mediate internalization on monocytes and neutrophils in the presence or absence of complement, reasoning that multimerization might form bigger complexes, increase complement activation, or improve Fc $\gamma$ R cross-linking. We found that in the absence of complement, neutrophils were slightly better, albeit not significantly, at internalizing HIV-1 opsonized by either monomeric or multimeric VRC01 than were monocytes. Moreover, on both monocytes and neutrophils, multimeric VRC01 was slightly more effective in mediating internalization than was monomeric MAb. In all cases absent complement, internalization remained at low levels, with a maximum 2-fold increase in internalization over a non-HIV-1-specific negative-control MAb observed with multimeric VRC01 added to neutrophils. The addition of complement had little effect on uptake of monomeric VRC01-opsonized HIV-1. However, complement markedly enhanced the ability of multimeric VRC01 to mediate uptake of virions on both monocytes and neutrophils.

In accordance with our previously published observations using monomeric MAbs in the absence of complement (9), uptake of opsonized virions by monocytes was low even though antibodies were fully capable of capturing HIV-1<sub>IGFP/JRFL</sub> virions. We now observe slightly increased internalization with neutrophils compared to monocytes. Nonetheless, the level of antibody-mediated internalization by neutrophils remains quite low. This inefficient uptake is perhaps best appreciated by comparing uptake of monomeric or multimeric VRC01-opsonized virions with the uptake of immune complexes formed by adding a goat anti-human antibody to virions opsonized with either NIH45-45 G54W (Fig. 2 and Fig. 3) or with monomeric VRC01 (Fig. 8). The large aggregates formed in this manner (9) result in an over 200-fold increase in uptake on neutrophils compared to the negative antibody control. In any case, it is possible that the increased uptake of NIH45-45 G54W<sub>IC</sub>- and VRC01<sub>1C</sub>-opsonized virions by neutrophils compared with monocytes is a result of the unique repertoire and higher expression levels of Fc gamma receptors, including Fc $\gamma$ RIIA, on the cell surface of neutrophils (16, 17). Furthermore, monocytes are composed of a variety of lineages and subsets whose activities may be unique at each stage of maturation (15).

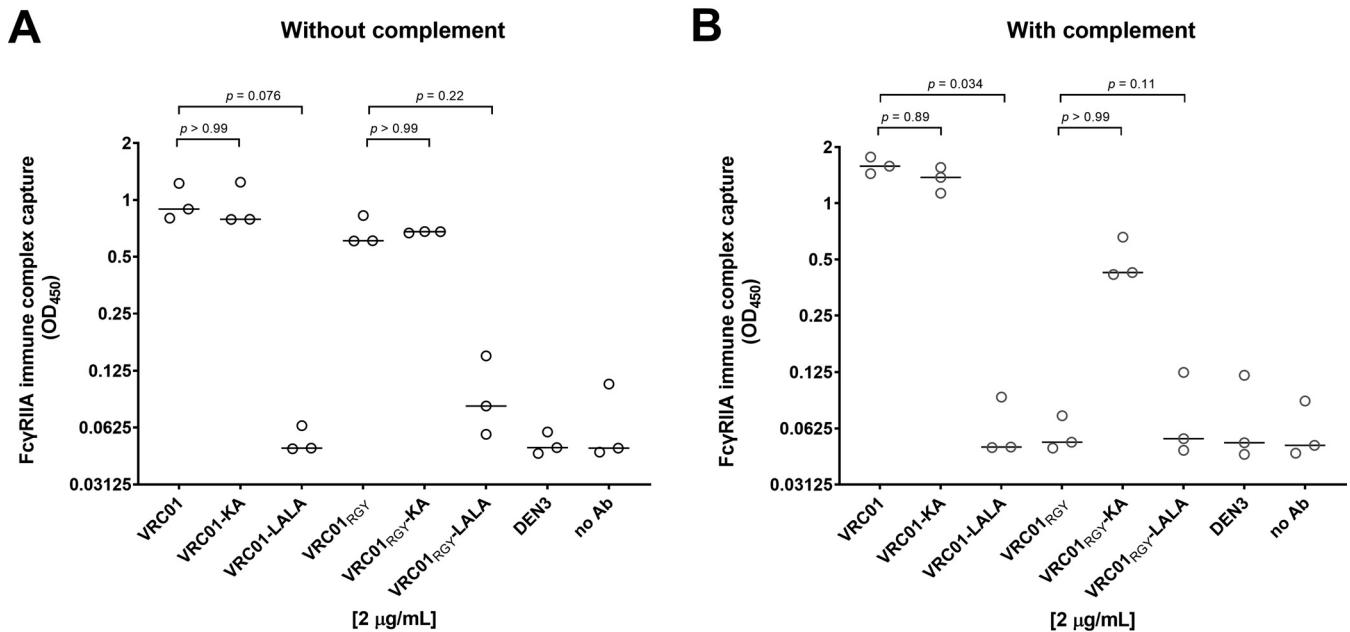


**FIG 9** Reduced complement binding results in a decrease in VRC01<sub>RGY</sub>-mediated virus internalization. (A and B) The VRC01 multimer variants VRC01<sub>RGY</sub>, VRC01<sub>RGY</sub>-KA, and VRC01<sub>RGY</sub>-LALA were tested for their ability to mediate HIV-1<sub>IGFP-JRFL</sub> internalization in monocytes (A) and neutrophils (B). Results are reported as virion internalization score (percent positive cells × MFI). Experiments (performed in triplicate) were repeated six times with cells from different healthy donors. Median internalization scores are indicated by a solid line. *P* values were calculated using Kruskal-Wallis with Dunn’s multiple-comparisons tests.

We observed only minimally improved internalization mediated by monomeric VRC01 after addition of complement. The limited effect of complement may be due to the fact that monomeric IgG1 only weakly interacts with a single globular head of the complement factor C1q hexamer (18).

It has been reported that antibody multimerization enhances effector functions, including antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity (12, 19). It is unknown whether such multimerization impacts antibody-mediated internalization of virus particles. In an attempt to develop a reagent capable of mediating more efficient phagocytic clearance of virus, we implemented an innovative Fc domain-multimerization approach using the CD4 binding site-specific MAb VRC01. In our hands VRC01<sub>RGY</sub> readily formed a high proportion of hexamers in solution as measured by SEC. This is in accordance with previous studies where introduction of the RGY triple mutation resulted in antibody hexamerization of other MAbs (14, 20). Although VRC01 hexamerization had no effect on gp140 binding in an ELISA, there was a slight increase in the ability of the multimers to capture HIV-1<sub>IGFP-JRFL</sub> virions compared with the monomers. Note that in all assays, monomeric and multimeric VRC01 were added in equal weight/volume concentrations so that the number of antigen-binding sites remained equivalent. The greater virion capture but equivalent gp140 binding by multimeric compared with monomeric VRC01 is likely the result of multivalency in the capture assay, where a hexamer can interact with one or more virus particles. The presence of detergent (i.e., Tween 20) in the gp140 ELISA, however, most likely prevents multimerization of VRC01 (as seen with our SDS-PAGE analysis).

Compared to monomeric VRC01, multimeric VRC01 in the absence of complement resulted in slight improvements in virus uptake by both monocytes and neutrophils. However, the addition of complement to multimeric VRC01 resulted in a substantial increase in virion uptake by both cell types. Confirming a role for complement in the enhanced virus uptake, we found that both the KA mutation (which only reduces



**FIG 10** Interactions between Fc $\gamma$ RIIA and monomeric and multimeric VRC01 are differentially affected by complement. (A and B) Interactions of monomeric and multimeric VRC01/HIV-1<sub>IGFP-JRFL</sub> immune complexes with Fc $\gamma$ RIIA were measured in the absence (A) and presence (B) of guinea pig complement. Virus capture was evaluated by subsequent p24 ELISA. All VRC01 variants and nonspecific controls were tested at 2  $\mu$ g/mL. Experiments were performed in triplicate and repeated three times. Lines represent median OD<sub>450</sub> values. *P* values were calculated using Kruskal-Wallis with Dunn's multiple-comparisons tests.

complement binding [13]) and the LALA mutation (which reduces both complement and Fc $\gamma$ R binding [13, 21]) greatly lowered antibody-mediated internalization by the multimeric VRC01<sub>RGY</sub>. In addition, the KA mutation had a slightly greater impact on internalization than did the LALA mutation, consistent with the fact that the KA mutation's effect on complement binding is greater than that of the LALA mutation (13).

The increased uptake of HIV-1<sub>IGFP-JRFL</sub> virions by multimeric VRC01<sub>RGY</sub> in the presence of complement is likely C1q dependent, since C1q is the component of complement that binds to IgG and since the KA mutant decreases internalization (Fig. 9) (22). This is in accordance with the notion that VRC01<sub>RGY</sub> multimers represent clusters of IgG that are vital to induce the conformational change required to expose the C1q-binding site within the antibody Fc region (20, 23). Once bound, the C1q-antibody-virus complex can be readily internalized by phagocytes in a C3b-independent manner (24).

Based on our finding that binding of VRC01 multimers to Fc $\gamma$ RIIA was nearly completely abrogated in the presence of complement and that the abrogated binding was mostly reversed by the KA mutation, it is likely that complement sterically hinders Fc-Fc $\gamma$ RIIA interactions. Notably, the binding of monomeric VRC01 to Fc $\gamma$ RIIA was not negatively impacted by complement or by the KA mutation, consistent with the limited ability of monomeric IgG to engage complement as discussed above. Thus, although multimerization improves virus internalization by phagocytes in the presence of complement, there appears to be a trade-off resulting in less Fc $\gamma$ RIIA binding (Fig. 10). How this might translate *in vivo* remains to be seen.

As mentioned above, antibody multimerization enhances effector functions, including antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity (12, 19). It will be of interest to ascertain the overall effect of antibody hexamerization on the clearance of infected cells *in vitro* and on the clearance of infected cells and virions *in vivo* in animal models of HIV-1 infection. In addition, to further study the role of complement in antibody-mediated virus internalization, HIV-1 envelope-specific IgM multimers could prove useful. IgM can recruit complement more efficiently than IgG (23), and IgM does not engage Fc $\gamma$ Rs. It is also possible that lower-valency IgG multimers, including dimers, could be sufficient to augment internalization of virions in the

**TABLE 1** Primers used for VRC01 heavy-chain mutagenesis

Mutation	Primer name	Primer sequence (5' to 3')
KA	K322A sense	5'-gcaaggagtacaagtgcgcggtctccaacaaagccc-3'
	K322A antisense	5'-gggcttggtagaccgcgactgtactccttc-3'
LALA	L234A L235A_sense	5'-accgtgccagcacctgaagccgagggggaccg-3'
	L234A L235A_antisense	5'-cggccccccggttcagggtctgggacaggt-3'
RGY	E345R sense	5'-tgtacacctgtgtcttcgggctgcccttg-3'
	E345R antisense	5'-ccaaagggcagccccgaagaccacaggtgtaca-3'
	E430G sense	5'-ggtgtgcagagccccatgcatcacggag-3'
	E430G antisense	5'-ctccgtgatgcatgggctctgcacaacc-3'
	S440Y sense	5'-cccggagacagggagagatacttctcgtgtagtggt-3'
	S440Y antisense	5'-accactacgcagagaagtatctctccgtctccggg-3'

presence of complement. Finally, by affecting antigen uptake and presentation, it is possible that hexameric antibodies could enhance anti-lentiviral immune responses in infected animals and humans.

We note that although the multimeric VRC01 consisted primarily of hexamers, the monomers present may have had an adverse effect on virion internalization. A more uniform reagent consisting solely of hexamers might further enhance uptake.

In conclusion, we confirmed our previous finding that antibody-mediated internalization of free HIV-1 virions by monocytes is very limited. In addition, we found that uptake of opsonized virions by neutrophils is also limited, though somewhat better than that by monocytes. By multimerizing the neutralizing MAb VRC01, further augmentation in virion uptake was noted, but substantial improvements only occurred when complement was added to multimeric VRC01. *In vivo* studies will be needed to determine if multimeric neutralizing antibodies will be therapeutically useful.

## MATERIALS AND METHODS

**Ethical statement.** Peripheral blood from anonymous healthy donors was obtained expressly for this research (through the University of California, Irvine, Normal Blood Donors Program) with informed, written consent in accordance with the institutional review board at the University of California, Irvine.

**Reagents.** The following reagents were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 JRFL gp140 recombinant protein (BJRFL gp140CF) from Barton F. Haynes and Hua-Xin Liao, VRC01 MAb heavy-chain and VRC01 MAb light-chain expression vectors from John Mascola, HIV Gag-iGFP-JRFL expression vector from Benjamin Chen, and the HIV-1-specific antibodies, VRC01, 10E8, 4E10, 3BNC117, NIH45-46 G54W, CH01, and HIVIG. The control human IgG1 monoclonal antibody (MAb), DEN3 (anti-dengue NS1), was provided by Dennis Burton (13, 25).

**Antibody mutagenesis.** The VRC01 heavy-chain expression vector was used as a DNA template for generating all VRC01 heavy-chain variants described in this study, including VRC01<sub>RGY</sub>, VRC01<sub>RGY</sub>-KA, VRC01<sub>RGY</sub>-LALA, VRC01-KA, and VRC01-LALA. Mutagenesis primers are listed in Table 1. Numbering of the amino acid positions in the antibody Fc region is according to the European Union numbering system (26). All amino acid substitutions, K322A, L234A, L235A, E345R, E430G, and S440Y, were introduced by site-directed mutagenesis using the QuikChange XL mutagenesis kit according to the manufacturer's instructions (Agilent). Amino acid substitutions of each VRC01 heavy-chain expression vector variant were confirmed by Sanger sequencing (Genewiz) prior to antibody expression and purification.

**Antibody expression and purification.** Adherent human embryonic kidney (HEK) 293T cells were cultured in serum-free medium (BalanCD HEK293, 1× Pen/Strep, 20 mM glutamine, and 1× insulin-transferrin-selenium) prior to transfection. Cells were cotransfected with VRC01 heavy- and light-chain-plasmid/polyethylenimine (PEI) complexes at a DNA/PEI ratio of 1:3. Four days posttransfection, antibody-containing supernatants were collected, cleared (2,500 rpm for 5 min), filtered (0.45 μm), and concentrated with a 50-kDa Amicon Ultra-15 centrifugal filter device. VRC01 variants were then purified over a protein A Sepharose spin column (Thermo Scientific) as previously reported (27). Eluted fractions containing the purified VRC01 antibody variants were concentrated, and buffer was exchanged using Amicon Ultra 0.5 mL centrifugal filter (50 kDa) units (Millipore). Antibody concentrations were measured at an optical density of 280 nm (OD<sub>280</sub>) with a NanoDrop spectrophotometer (Thermo Scientific).

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).** VRC01 antibody variants were separated by SDS-PAGE as previously described (28). Antibodies were examined under nonreducing (7.5% [wt/vol]) and reducing conditions (10% [wt/vol]) followed by a SimplyBlue SafeStain (Thermo Fisher) Coomassie G-250 staining procedure according to the manufacturer's instructions.

**Size exclusion chromatography (SEC).** For SEC analysis, a Superdex 200 increase 10/300 GL column (GE Healthcare) was washed at room temperature with a down-flow of 2.0 column volumes (CV) of 0.22-μm-filtered, sterile water at 0.500 mL/min. Next, the column was equilibrated with 2.0 CV of 0.22-μm-filtered, sterile phosphate-buffered saline (PBS), pH 7, at 0.750 mL/min. All VRC01 samples were filtered by

centrifugation through a 0.22- $\mu$ m cellulose acetate filter (Costar) at  $10,000 \times g$ , for 1 min at room temperature prior to loading onto a 0.1-mL sample loop (GE Healthcare). After a brief column wash with 0.05 CV of PBS at 0.750 mL/min, the sample loop was emptied with 2.00 mL of PBS at 0.750 mL/min onto the column. The column was eluted with 1.0 CV of PBS at 0.750 mL/min, and any eluted proteins were detected at UV 214 nm. The first 0.1 CV of eluate was wasted, and the remaining 0.9 CV of eluate was collected into 200- $\mu$ L fractions in 96-deep-well plates. Between each sample application, the column was rewashed and equilibrated. For protein size estimation, the column was calibrated with a protein standard (Bio-Rad) containing thyroglobulin (669 kDa), gamma globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B<sub>12</sub> (1.35 kDa).

**Antibody enzyme-linked immunoassay (ELISA).** ELISA plates were coated overnight at 4°C with 250 ng/well of a goat anti-human Fc fragment. Plates were then washed three times with wash buffer (Dulbecco's phosphate-buffered saline [DPBS]-containing 0.05% Tween 20) and incubated for 1 h at 37°C with 5% blocking buffer (wash buffer containing 5% nonfat dry milk). After washing the plates three times, serial dilutions (1:5 in 1% blocking buffer) of VRC01 antibody variants starting at 1  $\mu$ g/mL were added. Plates were further incubated for 1 h at 37°C. Unbound antibodies were removed by three washes, and bound antibodies were detected with a horseradish peroxidase conjugated goat anti-human F(ab')<sub>2</sub> antibody. After 1 h of incubation at 37°C, plates were washed four times, developed with 3,3',5,5'-tetramethylbenzidine (TMB) substrate, and stopped with H<sub>2</sub>SO<sub>4</sub>. Optical densities (at 450 nm) were measured with a Synergy 2 plate reader (BioTek). The IgG concentrations of the VRC01 variants were calculated based on a standard curve generated with a parental VRC01 obtained from the NIH AIDS Reagent Program.

**HIV-1<sub>JRFL</sub> gp140-binding ELISA.** HIV-1<sub>JRFL</sub> gp140 ELISA was carried out as previously reported (29) with some modifications. In brief, ELISA plates (Costar) were coated with 100 ng/well of recombinant JRFL gp140. Plates were washed, blocked, and incubated with serial dilutions (1:4) of MAbs starting at a concentration of 5  $\mu$ g/mL. After 1 h at 37°C, unbound antibodies were removed, and bound antibodies were detected by a horseradish peroxidase (HRP)-labeled goat anti-human Fc gamma-specific conjugate (Sigma-Aldrich). Plates were washed, developed, stopped, and read as described above. All antibodies were tested in duplicate, and binding assays were performed three times. Equilibrium dissociation constants ( $K_d$ ) at half-maximum binding were calculated using GraphPad Prism 9.2 software.

**Virus production and infectivity.** Full-length molecular clones of HIV-1<sub>IGFP/JRFL</sub> were generated and tested for infectivity as previously reported (9). In brief, HEK 293T cells were transfected with the HIV-1 plasmid HIV Gag-IGFP-JRFL using PEI as a transfection reagent at a DNA/PEI ratio of 1:3. After 3 days, virus-containing cell culture-supernatant fluid was harvested, concentrated, and stored at -80°C. Infectious units per mL of HIV-1<sub>IGFP/JRFL</sub> virions were determined by virus titration on TZM-bl luciferase reporter cells. Luminescence in relative light units (RLU) was measured using a Synergy 2 microplate luminometer (BioTek).

**Virion capture assay.** HIV-1 capture assays were carried out as previously described (9). Briefly, ELISA plates were coated with 250 ng/well of a goat anti-human IgG gamma chain-specific antibody (Jackson ImmunoResearch). Plates were then washed, blocked, and further incubated with 100 ng/well of HIV-1-specific antibodies. After washing, HIV-1<sub>IGFP/JRFL</sub> (10 ng of p24/well) was added for 4 h, and the p24 content captured on the plate was directly measured with a p24 ELISA. Samples were analyzed in triplicate, and assays were repeated three times. Monomeric and multimeric VRC01 variants were compared using equal weight/volume concentrations to keep the number of available binding sites proportional.

**Fc gamma receptor IIA (Fc $\gamma$ RIIA) virion capture assay.** ELISA plates were coated with a mouse anti 6 $\times$ -His tag-specific antibody (250 ng per well). VRC01 variants (2  $\mu$ g/mL) were mixed with HIV-1 virions (10 ng of p24/well) diluted in medium with guinea pig serum (10%) or heat-inactivated guinea pig serum and incubated at 37°C for 30 min to form MAb-HIV-1 immune complexes. The immune complexes were further incubated for 90 min at 37°C with soluble His-tagged human Fc $\gamma$ RIIA (4  $\mu$ g/mL). Next, the immune complex/Fc $\gamma$ RIIA mixture was transferred into blocked (5% blocking buffer) and washed anti-His tag antibody-coated plates and incubated for 90 min at 37°C. Plates were then washed five times with DPBS. Bound HIV-1 virions were lysed with viral RNA lysis buffer and quantified by HIV-1 p24 ELISA. All antibodies were tested in triplicate. The assay was repeated three times.

**HIV-1 p24 ELISA.** The HIV-1 p24 content of all viral preparations was determined using the RETRO-TEK HIV-1 p24 antigen ELISA kit (ZeptoMetrix) following the manufacturer's instructions. Levels of p24 were calculated by a point-to-point algorithm.

**Human polymorphonuclear neutrophil isolation.** Human peripheral neutrophils were isolated as previously described (30). In brief, blood from healthy HIV-1-negative donors was layered onto neutrophil isolation medium (Lympholyte-Poly; Cedarlane). After centrifugation, neutrophil-containing layers were isolated, washed, and resuspended in red cell lysis buffer (Santa Cruz). Neutrophils were washed once more and resuspended in the respective assay medium (RPMI, Pen/Strep, 20 mM glutamine) with active or heat-inactivated (1 h at 56°C) 10% guinea pig serum for subsequent experiments. To inhibit neutrophil extracellular trap (NET) release, cells were pretreated with 50  $\mu$ M diphenyleneiodonium chloride (DPI) 30 min prior to phagocytosis assays. Neutrophils were used in the antibody-dependent internalization assays the same day as isolation.

**Human monocyte isolation.** Human peripheral blood mononuclear cells were isolated from healthy, HIV-1-negative donors with a density gradient medium (Lymphoprep) according to the manufacturer's instructions (StemCell). Monocytes were separated using human CD14 MicroBeads according to the manufacturer's instructions (Miltenyi). Positively selected monocytes were subsequently

resuspended in assay medium (RPMI, Pen/Strep, 20 mM glutamine) supplemented with active or inactive guinea pig serum (10%) and used the same day in antibody-dependent internalization assays.

**Antibody-dependent internalization assay.** Note that we use the term “internalization,” rather than “endocytosis” or “phagocytosis” to refer to the uptake of opsonized virus particles, since we do not know the size of the particles. Phagocytosis usually refers to the internalization of particles  $>0.5 \mu\text{m}$ , whereas endocytosis refers to the internalization of particles  $<0.5 \mu\text{m}$  (31). Internalization assays were performed as previously described with some minor modifications (9). Assay medium (RPMI, Pen/Strep, 20 mM glutamine) containing active or inactive guinea pig serum (10%) was used for all internalization assays. HIV-1<sub>IGFP/JRFL</sub> virions (20 ng p24/reaction) were opsonized for 120 min at 37°C with monoclonal (50  $\mu\text{g}/\text{mL}$ ) or polyclonal antibody preparations (200  $\mu\text{g}/\text{mL}$ ) in a 1:1 (vol/vol) ratio. For immune complex formation with MAbs NIH45-46 G54W (NIH45-46 G54W<sub>IC</sub>) and VRC01 (VRC01<sub>IC</sub>), 5  $\mu\text{g}$  of a goat anti-human Fc gamma fragment was added after 30 min to the respective wells. Subsequently, the antibody-opsonized HIV-1 virions were incubated with DPI-treated neutrophils or monocytes (150,000 cells per well) for 75 min at 37°C. After internalization, cells were washed three times with ice-cold DPBS and fixed in 4% paraformaldehyde (PFA). Neutrophils and monocytes were analyzed by flow cytometry on a NovoCyte flow cytometer (ACEA) for internalized HIV-1<sub>IGFP/JRFL</sub> virions. A virion internalization score was determined by gating the samples on events representing live cells and calculating as follows: percent green fluorescent protein (GFP) positive  $\times$  median fluorescence intensity (MFI) GFP positive. Virus internalization experiments were performed at least five times with antibody samples tested in triplicate.

**Statistical analysis.** All statistics were performed using Graph Pad Prism 9.2. For paired-group analyses, we used Wilcoxon matched-pair signed rank tests. For unpaired group analyses, we used Mann-Whitney rank tests. For multiple-group comparisons, Kruskal-Wallis tests followed by Dunn’s correction were used.

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