Binding of the mannose-specific lectin, griffithsin, to HIV-1 gp120 exposes the CD4-binding site

Running Title: GRFT exposes the CD4-binding site

Kabamba Bankoledi Alexandre[1], Elin S Gray[1], Ralph Pantophlet[2,3], Penny L Moore[1], James B McMahon[4], Ereck Chakauya[5], Barry R O'Keefe[4], Rachel Chikwamba[5] and Lynn Morris[1*]

[1]AIDS Virus Research Unit, National Institute for Communicable Diseases, Johannesburg, South Africa; [2]Faculty of Health Sciences and [3]Department of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, Canada; [4]Molecular Targets Laboratory, Center for Cancer Research, NCI-Frederick, Maryland, USA and [5]Centre for Scientific and Industrial Research, Pretoria, South Africa.

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*Corresponding author mailing address: National Institute for Communicable Diseases, Johannesburg, Private Bag X4, Sandringham 2131, Johannesburg, South Africa. Phone: +2711-386-6332. Fax: +2711-386-6453. E-mail: lynnm@nicd.ac.za.

ABSTRACT

The glycans on HIV-1 gp120 play an important role in shielding neutralization-sensitive epitopes from antibody recognition. They also serve as targets for lectins that bind mannose-rich glycans. In this study we investigated the interaction of the lectin griffithsin (GRFT) with HIV-1 gp120 and its effects on exposure of the CD4-binding site (CD4bs). We found that GRFT enhanced the binding of HIV-1 onto plates coated with anti-CD4bs antibodies b12, b6 or the CD4 receptor mimetic, CD4-IgG2. The average enhancement of b12 or b6 binding was higher for subtype B viruses compared to subtype C while for CD4-IgG2 it was similar for both subtypes, although lower than observed with antibodies. This GRFT-mediated enhancement of HIV-1 binding to b12 was reflected in synergistic neutralization for 2 of the 4 viruses tested. The glycan at position 386 which shields the CD4bs was involved in both GRFT-mediated enhancement of binding as well as neutralization synergism between GRFT and b12. Although GRFT enhanced CD4bs exposure, it simultaneously inhibited ligand binding to the coreceptor binding site, suggesting that GRFT-dependent enhancement and neutralization utilize independent mechanisms. This study shows for the first time that GRFT interaction with gp120 exposes the CD4bs through binding the glycan at position 386 which may have implications for how to access this conserved site.

INTRODUCTION

HIV-1 gp120 is heavily glycosylated and N-linked glycans account for ~50% of its molecular mass (30, 32). Three types of glycans are found on gp120; namely, high-mannose glycans composed of 7 to 9 terminal mannose residues, complex glycans containing terminal sialic acid residues and hybrid glycans which are a mixture of both (9, 20, 29, 62). There are approximately 11 glycosylation sites on monomeric gp120 that are occupied by either mannose-rich or hybrid glycans, while the remaining sites bear complex glycans (32). However, recently Doores *et al.* reported that 98% of glycans on native HIV-1 envelope (Env) are barely processed beyond $Man_5GlcNAc_2$ i.e. glycans containing five mannose residues (16). Glycosylation patterns between HIV-1 subtypes B and C envelopes have also been reported to differ in number and frequency (60). In addition to their role in promoting the proper folding of gp120 and mediating its interaction with cellular receptors, glycans protect HIV-1 from antibody neutralization by masking sensitive epitopes on the envelope (19, 34, 35, 37-39, 54, 55).

The CD4 binding site (CD4bs) on gp120 is highly conserved amongst HIV-1 subtypes and is a target for antibodies (29, 58). Amongst HIV-1 antibodies that target the CD4bs is the broadly neutralizing monoclonal antibody b12. The epitope of this antibody is located primarily in the neutralizing face of gp120 and 82% of its binding site is in the outer domain of the viral envelope (61). However, the high-mannose glycan at position 386 located inside the CD4bs shields this site from antibodies as its removal has been shown to increase HIV-1 sensitivity to b12 (24, 48, 52, 66). In addition, the CD4bs binding site is a target of non-neutralizing antibodies such as b6. However, unlike b12 that binds both monomeric and trimeric gp120, b6 binds only to the monomeric form of this glycoprotein (48).

High-mannose glycans on gp120 are also targets of glycan-specific agents such as lectins. Several lectins have been identified in recent years that potently block the infectivity of viruses such as HIV and influenza (6, 41, 46). One of the most potent of these is griffithsin (GRFT). GRFT is a 121 amino acids and ~13 kDa molecular weight lectin that was originally isolated from the red alga *Griffithsia* sp. (41). GRFT exists exclusively as a dimer and has a domain swapped structure in which two β-strands of one monomer combine with 10 β-strands of the other monomer to form a β prism of three four-stranded sheets (63, 64). Each GRFT

monomer contains three binding sites that have high affinity for mannose residues. Both native and recombinant GRFT display potent antiviral activities against primary HIV-1 isolates by binding to high-mannose glycans on the viral envelope spike (41, 47). We previously showed that the 234 and 295 glycosylation sites play an important role in GRFT neutralization of HIV-1 (1).

Since GRFT binds high-mannose oligosaccharides, including the one at position 386 that conceals the b12 epitope, we wished to explore whether this lectin affected exposure of the CD4bs. We examined both binding using a virus capture assay as well as neutralization. We found that GRFT enhanced HIV-1 binding of b12 and the non-neutralizing CD4bs mAb b6 as well as CD4-IgG2, which was used here as a surrogate for the CD4 receptor molecule. Importantly, GRFT and b12 synergised to render some HIV-1 isolates more sensitive to neutralization. The glycan at position 386 on gp120 was found to play a role in both enhancement and synergy suggesting that GRFT could be used to increase exposure of the CD4bs of HIV-1.

MATERIALS AND METHODS

Viruses and reagents: HIV-1 subtype B envelope clones QH0692.42 and PVO.4 amplified from acutely infected individuals (33) were obtained from the NIH Reference and Reagent Program. The cloned subtype C envelopes COT9.6 and COT6.15 were derived from chronic paediatric infections (21), while Du151.2 and CAP239.G3J were amplified from acutely infected patients (23, 56). Infectious primary viruses were isolated from subtype B (DS12) and subtype C (Du151, CM9) infected adults (12, 13) while RP1 was isolated from a chronic paediatric patient (21). The HIV-2 envelope clone 7312A was provided by Dr George Shaw and the pSG3Δ*env* plasmid was obtained from Dr Beatrice Hahn. The mAbs IgG1b12 (b12) and IgG1b6 (b6) were kindly provided by Dr Dennis Burton. MAbs 4E10, F240 and 17b were obtained from the NIH Reference and Reagent Program. The subtype C specific anti-V3 mAb, 3468L was isolated from an HIV-positive patient (22). The anti-CCR5 inhibitor PRO140, the CD4 receptor surrogate CD4-IgG2 and the soluble CD4 (sCD4) were generously provided by Progenics Pharmaceuticals, Inc. (Tarrytown, NY). Recombinant GRFT and CV-N were purified from *E. coli* at the National Cancer Institute, MA, USA (6, 41).

Generation of env-pseudotyped viruses: HIV-1 pseudoviruses were generated by co-transfection of the gp41-gp120 (Env) and pSG3Δ*env* plasmids (55) into 293T cells using the Fugene transfection reagent (Roche Applied Science, Indianapolis, IN). The $TCID_{50}$ of each virus stock was determined by infecting TZM-bl cells with serial 5-fold dilutions of the supernatant in quadruplicate in the presence of DEAE dextran (37.5 μg/mL) (Sigma-Aldrich, St. Louis, MO). The Bright Glo™ Reagent (Promega, Madison, WI) was used to measure infection after 48 hours of culture, according to the manufacturer's instructions. Luminescence was measured in a Wallac 1420 Victor Multilabel Counter (Perkin-Elmer, Norwalk, CT). The $TCID_{50}$ values were calculated as described elsewhere (27).

Site-directed mutagenesis: N-linked glycosylation site signal sequences were introduced in HIV-1 gp120 by QuikChange Site Directed Mutagenesis (Stratagene, LaJolla, CA). The presence of the mutation was confirmed by sequencing using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied

Biosystem, Foster City, CA) and resolved on the ABI 3100 automated genetic analyzer.

Single cycle neutralization assay (TZM-bl assay): Pseudovirus neutralization assays were performed as described elsewhere (40). Briefly, a 3-fold dilution series of GRFT in 100 µL of DMEM with 10% FBS (growth medium) was prepared in a 96-well plate in duplicate. Two hundred $TCID_{50}$ of pseudovirus in 50 µL of growth medium was added and the mixture was incubated for 1 hour at 37ºC. Then 100 µL of TZM-bl cells at a concentration of $1x10^5$ cells/mL containing 37.5 µg/mL of DEAE dextran was added to each well and cultured at 37ºC for 48 hours. Infection was evaluated by measuring the activity of the firefly luciferase. Titres were calculated as the inhibitory concentration that causes 50% reduction (IC_{50}) of relative light unit (RLU) compared to the virus control (wells with no inhibitor) after the subtraction of the background (wells without both the virus and the inhibitor).

To measure synergism, HIV-1 was incubated with a 3-fold dilution series of GRFT and b12 in 100 µL of DMEM-10% FBS. The lectin and the mAb were diluted alone and in combination. This was followed by the addition of the virus to TZM-bl cells. The IC_{50} and IC_{80} of GRFT and b12 were determined in the wells containing each compound alone and in the wells containing a mixture of both compounds. Synergism between GRFT and HIV-positive plasma or serum was measured as for b12 except that here ID_{50} and ID_{80} were calculated. Since PRO140 is a CCR5 inhibitor, TZM-bl cells were first incubated with a dilution series of this compound prior to the addition of the virus with or without GRFT (in a dilution series) to allow for PRO140 binding to the co-receptor. The IC_{50} and IC_{80} of the lectin and PRO140 when used alone and in combination were calculated. Synergism was determined by calculating the combination index (CI) using both IC_{50} and IC_{80} (11, 59). A CI of 0.3 to 0.7 was deemed indicative of synergism, 0.7 to 0.85 of moderate synergism, 0.85 to 0.9 of slight synergism and 0.9 to 1.1 of an additive effect as previously defined (11, 67).

GRFT inhibition of HIV-1 infection of U87-CCR5 and U87-CXCR4: U87-CCR5 or U87-CXCR4 at a concentration of $2x10^4$ cell/well were cultured for 24 hours in a flat bottom plate. This was followed by the addition of HIV-1 primary isolates that were pre-incubated for 1 hour with a 3-fold dilution series of GRFT in 150 µL of DMEM

with 10% FBS. The following day cells were washed three times and cultured for 10 days. The percentage inhibition of infection was determined by comparing the p24 concentration of GRFT containing wells to the control wells. The IC_{50} of GRFT inhibition of HIV-1 was calculated by plotting the lectin concentration against the percentage inhibition in a linear regression using GraphPad Prism 4.0.

HIV-1 virion capture assay: A high binding 96-well plate (Corning Incorporated Corning, New York, U.S.A) was coated overnight with 100 µL/well of a 10 µg/mL solution of b12, b6, F240, 4E10, 3468L or CD4-IgG2 in $NaHCO_3$ (pH 8.5). The coated plate was washed three times with phosphate buffered saline (PBS) and blocked with 3% bovine serum albumin (BSA) in PBS at 37ºC for 2 hours. HIV-1 was incubated for an hour with GRFT, CV-N, CD4-IgG2 or the mAbs and the virus was then added to the plate and left at 37ºC for 2 hours. The plate was washed three times with PBS and captured virus was lysed with 150 µL of 0.5% Triton-X 100 and the p24 concentration was measured by ELISA. Control wells contained HIV-1 captured in the absence of the lectins, mAbs or CD4-IgG2.

This assay was modified to assess competition between GRFT and 17b for binding to the CD4 induced epitope (CD4i) using the HIV-2 virus 7312A in the presence of sCD4 (15). After 1 hour incubation with different concentrations of the lectin the virus was incubated with 25 µg/mL of sCD4 for another 1 hour before addition to a 17b-coated plate for 2 hours. After washing the plate three times with PBS the amount of captured p24 was quantified as explained above. In another version of the same experiment the virus was incubated first with 25 µg/mL of sCD4, then with different concentrations of the lectin before addition to the 17b-coated plate for 2 hours. For GRFT competition with 17b, using a plate coated with CD4-IgG2, the virus was first incubated with the CD4-IgG2-coated plate for 1 hour. This was followed by the sequential addition of 25 µg/mL of sCD4 (to expose the CD4i on Env spikes that didn't bind to the surface bound CD4-IgG2), then 30, 6 or 1.2 µg/mL of 17b and thereafter by 30 µg/mL of GRFT, each for 1 hour. The plate was washed with PBS three times between incubations. Rabbit anti-GRFT polyclonal antibodies and an HRP-conjugated goat anti-rabbit antibody were used to measure the amount of GRFT bound to the virus using an optical density of 450 nM.

RESULTS

HIV-1 binding to b12 and b6 mAbs was enhanced in the presence of GRFT

We previously showed that GRFT competes with the glycan-specific mAb 2G12 for binding to HIV-1 (1). During this investigation we observed that GRFT enhanced the binding of another anti-HIV mAb b12, which targets the CD4 binding site (CD4bs) and is broadly neutralizing (4, 5). In order to further explore this observation we made use of a virus capture assay (VCA) that is highly reproducible and commonly used (8, 10, 45, 50). Most importantly VCA offers a simple way of evaluating HIV-1 interaction with both neutralizing and non-neutralizing antibodies as both capture viruses (10), although some gp41 antibodies have been reported to be capture only weakly (31). However, this assay has the disadvantage of being unable to provide information about the neutralization capacity of the antibody studied and so in this study we performed both VCA and neutralization in parallel. We used two subtype B (QH0692.42 and PVO.4) and four subtype C (COT6.15, COT9.6, Du151.2 and CAP239.G3J) pseudoviruses which were pre-incubated with GRFT and then added to b12-coated plates. Captured viruses were lysed and the amount of p24 antigen measured relative to wells with no lectin. As shown in Figure 1A, pre-incubation with GRFT significantly enhanced viral capture by the b12 mAb above control, b12-coated wells with no GRFT. The greatest effect was observed with the 2 subtype B viruses; for PVO.4 it was 8-fold above the control and for QH0692.42 it reached 4-fold. The effect was slightly less pronounced for the four subtype C viruses, for Du151.2 and COT6.15 binding increased by 4-fold while for CAP239.G3J binding increased by 2-fold and for COT9.6 the increase was 1.5-fold above the control.

In order to determine if this GRFT enhancement of binding was specific to the b12 mAb, we tested another CD4bs mAb called b6, which is non-neutralizing despite its epitope overlapping with that of b12 (48, 51). GRFT enhanced the binding of all six viruses to b6 and the effect was even more striking compared to b12 (Figure 1B). Again the subtype B viruses showed higher levels of binding compared to the subtype C viruses. The subtype B virus QH0692.42 exceeded the control by 13-fold while among subtype C viruses CAP239.G3J was the highest reaching 6.2-fold of the control. For both b12 and b6, most viruses reached the maximum enhancement between 0.2 and 1 μg/mL of GRFT suggesting saturation of CD4bs exposure with

little additional effect at higher concentrations. In most cases a reduction in binding was observed for both b12 and b6 at 0.04 μg/mL, indicating a dose response effect. CAP239.G3J reached maximal enhancement at the lowest GRFT concentration tested perhaps because of its unusual sensitivity to GRFT compared to other subtype C viruses (see Table 5). Both b12 and b6 competed against themselves as expected (Figure 1A and 1B).

Since the CD4bs overlaps both the b12 and b6 epitopes we also studied whether GRFT increased CD4 receptor binding to HIV-1 gp120. To achieve this we carried out a capture assay using the chimeric molecule CD4-IgG2 and the viruses QH0692.42 and COT6.15 that showed the highest enhancement of b12 and b6 binding. As shown in Figure 2 the presence of GRFT modestly enhanced the capture with CD4-IgG2 and this enhancement reached ~ 2-fold of the control for both viruses.

Enhancement of b12 and b6 binding was specific to GRFT and does not involve HIV-1 virion cross-linking

In order to explore the specificity of the observed enhanced exposure of the CD4bs by GRFT, we turned to CV-N, another mannose-specific lectin with strong anti-HIV neutralizing activity (64). QH0692.42 and COT6.15 were captured with either b12 or b6 in the presence of CV-N. Contrary to the observations with GRFT, CV-N blocked virus capture by both antibodies (Figure 3A-D) showing that the ability of GRFT to enhance the binding of b12 and b6 to HIV-1 was specific to this lectin.

Next we determined whether the GRFT-mediated enhancement of b12 and b6 binding was the result of cross-linking of viral particles via the mannose residues. If so, similar enhancement should be observed if b12 or b6 were substituted for another antibody whose epitope does not overlap the CD4bs. We selected the mAbs F240 and 4E10 that bind gp41 at the immunodominant epitope and the membrane proximal external region (MPER), respectively (7, 36) and 3468L, a mAb that binds the V3 loop (22) as the substitute antibodies. These were coated onto an ELISA plate and the pseudoviruses QH0692.42, PVO.4 and Du151.2 which had been pre-incubated with different concentrations of GRFT were added. We observed that unlike b12 and b6, GRFT had no effect on virus binding to F240 and 4E10-coated plates and p24 levels were equivalent to control wells without GRFT (Figure 4A and 4B show data for 1 representative virus). There was also no enhanced binding of viruses to the anti-V3 mAb-coated plate, but unexpectedly GRFT competed with 3468L for binding to these

viruses (Figure 4C). Nevertheless, these data indicated that the GRFT mediated enhancement of binding to gp120 is specific to CD4bs antibodies and does not involve HIV-1 virion cross-linking by the lectin.

The glycan at position 386 was involved in GRFT mediated enhancement of HIV-1 binding to b12 and b6

In order to determine if the number or position of mannose-rich glycans influenced the GRFT-mediated enhancement of b12 and b6 binding, we compared the glycosylation sites of all the viruses used in this study (Table 1). We observed that except for COT9.6 and Du151.2 that lacked one and three glycans respectively, all viruses lacked two glycosylation sites, suggesting that the number of glycans was not responsible for the variance in enhancement observed with these viruses. Three of the 4 subtype C viruses lacked the glycan at position 295 which is typical of viruses from this subtype (60). All six viruses, however, had intact glycosylation sites at positions 241, 262, 332 and 386.

We and others have previously shown that removal of the glycan at position 386 in HIV-1 gp120 increases the virus sensitivity to b12 neutralization (17, 24, 52). We therefore investigated whether this glycan might be involved in the GRFT mediated enhancement of b12 and b6 binding to HIV-1. We deleted this site in PVO.4 and QH0692.42 and in both cases this deletion resulted in a roughly 2- fold decrease in GRFT mediated enhancement of binding to b12 and b6 (Table 2) suggesting that the 386 glycosylation site was involved in this GRFT mediated effect.

Since subtype C viruses showed lower levels of GRFT-mediated enhancement of b12 and b6 binding compared to subtype B, we reconstituted all the mannose-rich glycans on COT6.15 and COT9.6 to determine if this increased the levels of binding. Addition of the 295N and 448N glycans to the COT6.15 envelope resulted in ~2-fold higher enhancement of binding to b6 while for COT9.6 addition of 295N increased both b6 and b12 binding compared to the corresponding wild-type viruses. Confirming what was seen for the 2 subtype B viruses, deletion of the 386 glycosylation site in these reconstituted subtype C viruses resulted in a similar decrease in the enhancement of b12 binding (Table 2).

GRFT synergized with b12 to inhibit HIV-1 infection

To determine whether GRFT enhancement of b12 binding to HIV-1 translated into a synergistic interaction between these two compounds, we infected TZM-bl cells in the presence of GRFT and b12, individually and in combination. PVO.4 and CAP239.G3J were not neutralized by b12 and the presence of this mAb did not affect their sensitivity to GRFT, so synergy could not be measured. Viral infection was quantified after 48 hours and synergism between the antibody and the lectin at IC_{50} and IC_{80} was measured by calculating the combination index (CI) (59). Both GRFT and b12 when tested individually neutralized all four pseudoviruses (Table 3). We observed synergism between b12 and GRFT for the neutralization of QH0692.42 and COT6.15 but not for COT9.6 or Du151.2. The CI values for QH0692.42 (at GRFT : b12 mole ratio of 1.2 : 1) were 0.6 at IC_{50} and IC_{80} and for COT6.15 (at GRFT : b12 mole ratio of 1 : 16.5) it was 0.7 at $IC_{50.}$ We were not able to calculate the CI value at IC_{80} for COT6.15 as the highest inhibition of this virus with b12 was 65% (data not shown). Lastly, we tested for synergism between GRFT and b6 for the neutralization of the six viruses mentioned above and we observed no synergism (data no shown). In addition, the presence of b6 did not affect these viruses sensitivity to the lectin (data not shown).

Since we showed that the glycan at position 386 was involved in the GRFT-mediated enhancement of binding we investigated its effect on the observed synergy. For this experiment we used QH0692.42 and the COT6.15 virus reconstituted with the 295N and 448N glycans which showed the same level of synergy as the parental virus (Table 3). The N386Q mutant in this reconstituted virus was, however, considerably more sensitive to b12 as previously reported (Table 3) (24). For both QH0692-N386Q and COT6-V295N/S448N/N386Q there was a loss of synergism between GRFT and b12 at IC_{50}, implicating the 386 glycan in the synergistic interaction between GRFT and b12 for neutralization of the viruses tested.

To determine which compound benefited the most in this synergistic interaction, we analyzed the shift in the inhibition curves for GRFT and b12, when used alone or in combination. As shown in Figure 5A and 5B, the combination of GRFT and b12 shifted the inhibition curves to the left relative to the curves for the single compounds. This suggested that QH0692.42 and COT6-V295N/S448N were more sensitive to each compound when used in combination. These observed shifts while not statistically significant were consistent. For b12 the IC_{50} values were 13-

fold lower in the presence of GRFT while for GRFT they were 2-fold lower suggesting that b12 benefited the most from this combination. The deletion of the 386 glycan in both viruses resulted in a loss of synergy. This was shown by overlapping of the curves for GRFT when used either singly or in combination with b12 (Figure 5C and 5D). In agreement with what we observed previously, the effect of the removal of the 386 glycan on GRFT neutralization curves was consistent but minor and not statistically significant. The curves for b12 were also affected; thus for QH0692-N386Q there was a 63% reduction in the shift between the single and combination curves for b12, while for COT6-V295N/S448N/N386Q there was a 40% reduction despite the increased sensitivity of this virus to b12. These data confirm the involvement of the 386 glycan in GRFT and b12 synergistic interaction.

Synergy between GRFT and anti-CD4bs plasma antibodies

Since plasma from some HIV-infected individuals contain antibodies that target the CD4bs (25), we investigated whether GRFT affects ID_{50} titres for the neutralization of HIV-1. We used HIV-positive plasma from BB10 that was shown to contain antibodies to the CD4bs and compared this to BB34 that contained anti-MPER antibodies (25). TZM-bl cells were infected with HIV-1 in the presence of GRFT with or without plasma starting at a 1:20 dilution. Only the CI at ID_{50} was measured as the highest neutralization with both plasmas was below 80%. GRFT and BB10 acted synergistically to neutralize all 4 viruses tested with CI values ranging from 0.2 – 0.7. In contrast, GRFT and BB34 synergized to neutralize only CAP239.G3J with a CI of 0.4 while there was antagonism for QH0692.42 (Table 4).

CAP239.G3J showed synergy with both heterologous plasmas and so we examined GRFT in combination with the autologous serum. However, we observed no synergism between the lectin and the serum (data not shown) consistent with the observation that it did not contain anti-CD4bs antibodies (Mascola, unpublished data). In conclusion these data show that that GRFT can increase HIV-1 sensitivity to plasma containing CD4bs antibodies.

GRFT inhibited sCD4-induced 17b binding and blocked infection via both CCR5 and CXCR4

Given that GRFT inhibits HIV infection by binding glycans and blocking receptor engagement, we asked how this lectin might promote virus binding to the CD4

receptor while inhibiting viral infection. We hypothesized that GRFT inhibits HIV infection by blocking steps that follow the CD4 receptor binding event, such as the co-receptor binding step. To investigate this possibility, we used the HIV-2 pseudovirus 7312A, which following incubation with soluble CD4 becomes sensitive to the 17b mAb that binds to CD4 induced (CD4i) epitopes (15). First we established that 7312A sensitivity to GRFT was similar to HIV-1 by carrying out a neutralization assay in TZM-bl cells. GRFT inhibited this virus with an IC_{50} of 4 nM which is comparable to what we obtained with HIV-1 (1). We incubated 7312A with GRFT and then with sCD4 prior to capture with 17b. We also reversed the order i.e. we incubated the virus with sCD4 first before incubation with GRFT prior to capture onto the ELISA plate. As shown in Figure 6A and 6B, GRFT reduced the amount of virus captured with 17b in the presence of sCD4 irrespective of when sCD4 was added, suggesting that GRFT inhibits the interaction of 17b with the co-receptor binding site.

To determine if 17b interfered with GRFT binding, we added 7312A to an ELISA plate coated with CD4-IgG2, then sequentially added sCD4, 17b and GRFT to the plate before adding rabbit anti-GRFT polyclonal antibodies. Figure 6C shows that the increase in the concentration of 17b had no effect on GRFT binding to the virus. Similarly, GRFT binding to an ELISA plate coated with monomeric gp120, did not reduce 17b binding (data not shown) confirming that GRFT does not interfere with 17b binding. Taken together these data indicate that GRFT and 17b do not occlude each others binding sites i.e. there is no steric obstruction between these two compounds.

Given the possibility that GRFT interferes with the co-receptor binding to HIV-1, we analyzed whether lectin inhibition of the virus was co-receptor specific. U87 cells expressing either the CCR5 or CXCR4 co-receptor were infected with dual tropic infectious HIV-1 viruses Du151, DS12, CM9 and RP1 in the presence of GRFT. As shown in Figure 7, GRFT inhibited infection in both cell lines with similar IC_{50} values regardless of which co-receptor the virus used more efficiently.

Since different classes of entry inhibitors have been shown to act synergistically to inhibit HIV-1 infection (43, 44, 53, 65), we next determined whether the anti-CCR5 antibody PRO140 synergized with GRFT to inhibit HIV-1 infection. Six pseudoviruses were tested at GRFT and PRO140 mole ratio of 1 : 50. As shown in Table 5, four viruses showed moderate synergy between GRFT and PRO140 of 0.6-0.7 at IC_{80}. This suggested that while GRFT and the anti-CCR5 mAb

PRO140 can synergise to inhibit HIV-1 infection, this effect requires high concentrations of both compounds. In addition, since we previously showed that GRFT enhanced QH0692.42 and COT6.15 binding to CD4IgG2 (Figure 2), we investigated whether this lectin synergized with sCD4 to neutralize these viruses. We found that there was synergism between GRFT and sCD4 for the neutralization of QH0692.42 with CI of 0.7 and 0.6 at IC_{50} and IC_{80}, respectively.

DISCUSSION

In this study we have shown that GRFT enhanced HIV-1 binding to the CD4bs mAbs b12 and b6 and the CD4 surrogate CD4-IgG2. The observed enhancement was specific to GRFT as no effect was seen with CV-N, another mannose-binding lectin. The enhancement of binding with GRFT was not due to lectin cross-linking of viruses as HIV-1 binding to gp41 and V3 specific mAbs were not affected. This enhanced binding resulted in moderate synergistic neutralization between b12 and GRFT for some viruses. The high-mannose glycan at position 386 on gp120 was implicated in both the enhancement and synergism between GRFT and b12 probably because this glycan shields the CD4bs (18). Lastly, in this study we also showed that GRFT inhibits CCR5 and CXCR4-mediated HIV-1 infection with similar potency and that this lectin can synergizes with plasma from HIV-positive individuals and with the anti-CCR5 inhibitor PRO140 to neutralize HIV-1.

GRFT enhancement of HIV-1 binding was higher for subtype B compared to subtype C viruses. Glycosylation differences in the envelopes of these two subtypes may explain this (60) although a previous study suggested that these differences had no impact on neutralization by GRFT (1). Another possible reason is that subtype B viruses show a higher affinity for b12 compared to subtype C. Indeed subtype B viruses are neutralized by b12 and b6 more frequently and more potently than subtype C viruses (5). However, this is unlikely to be the only reason as PVO.4, which is resistant to b12, showed the highest levels of b12 binding in the presence of GRFT. Clearly, a larger number of viruses from both subtypes should be tested to determine whether substantial differences in GRFT-mediated enhancement of b12 and b6 binding exist.

In general, b6 binding was higher than b12 suggesting that the b6 epitope may be more affected by the GRFT mediated exposure of the CD4bs. GRFT also increased binding of CD4-IgG2 although the enhancement was lower than for the anti-CD4bs antibodies of the 2 viruses tested. This may be due to the fact that the CD4 receptor binds further away from glycan 386 or because it is simply less affected by GRFT binding to gp120. However, the use of antibodies and the CD4 mimetic provides compelling evidence that GRFT exposes the entire CD4bs and not just the binding sites of the antibodies.

In 2 of the 4 viruses tested, GRFT-mediated enhanced binding to b12 resulted in synergistic neutralization between GRFT and b12. The individual virus sensitivity

to GRFT and b12 neutralization as well as the level of GRFT-mediated enhancement of binding to b12 are likely to be important determining factors for neutralization synergy to occur. Thus QH0692.42 and COT6.15, the 2 viruses where synergy was observed, showed the highest levels of enhancement and both were sensitive to GRFT and b12 neutralization. However, for COT6.15 the ratio of b12 to GRFT used to achieve synergism was very high, probably because high levels of b12 were required for COT6.15 neutralization. The lack of synergy for COT9.6 may be explained by the comparatively low levels of enhancement of b12 binding in the presence of GRFT. The Du151.2 virus did not show synergism despite being sensitive to b12 and GRFT and its enhancement was similar to COT6.15, suggesting that other factors were involved; for example this virus was the only one that lacked the 339 glycan. Two viruses were not sensitive to b12 and so synergy could not be assessed; PVO.4 probably due to trimer-shielding as this virus had all the required b12 contact residues while in CAP239.G3J resistance to b12 was due to sequence variation at these sites (61). Although no synergism was noted between GRFT and b6 as expected, given it is a non-neutralizing antibody, the lectin acted synergistically with sCD4 to neutralize QH0692.42. This suggested that like the enhancement of binding, GRFT-mediated synergy involved more than just the b12 binding site. Furthermore, synergy between GRFT and HIV-positive plasma BB10, which contains anti-CD4bs antibodies (25), for all the viruses tested, is consistent with GRFT exposure of the CD4bs. However, the lack of synergistic interaction between the lectin and BB34 for 3 of these viruses is in agreement with the fact that this plasma contains antibodies that target the MPER (25). This supports data shown in Figure 4 that shows GRFT does not enhance HIV-1 binding to 4E10.

We also observed synergism at IC_{80} between GRFT and the CCR5 inhibitor PRO140 for the neutralization of HIV-1 in 4 of the 6 viruses tested. However, the mechanism of this interaction is most probably unrelated to that of the GRFT/b12 since the PRO140 mAb binds CCR5 on the cell surface thereby blocking co-receptor interactions, while the b12 mAb binds gp120 blocking the initial association with CD4. Synergy between PRO140 and other CCR5 inhibitors has been noted previously with CI values between 0.38 - 0.61 (43). This is similar to what is reported in this study which for GRFT in combination with PRO140, b12 or sCD4 was between 0.6-0.7 while for BB10 plasma it was 0.2 - 0.7. Comparison to CI that have been published for other HIV-1 inhibitors that includes a fusion inhibitor such as

AMD3100 and T20 (0,.03) and PRO542 and T20 (0.14), suggests that the synergistic interactions we observed with GRFT were moderate (44, 53).

The high-mannose glycan at position 386 is located within the CD4bs (29, 32, 48) and deletion of this glycan has been shown to increase HIV-1 neutralization sensitivity to b12 (24, 52). It is possible that the mechanism of GRFT-mediated enhancement of b12 binding and synergy is the result of GRFT binding to 386 and shifting this glycan to increase accessibility to the CD4bs. Removal of this glycan resulted in a decrease in b12 binding and also decreased synergy between GRFT and b12. Deletion of the 386 glycan probably impacted GRFT binding to gp120 as was suggested by QH0692.42 (Table 3). Duenas-Decamp *et al*. (17) showed that the 386 glycosylation site requires arginine at position 373 to modulate HIV-1 resistance to b12. However, given that none of the viruses we tested had arginine 373, this residue is most probably not important for the GRFT mediated enhancement of HIV-1 binding to b12.

CV-N, like GRFT, binds high-mannose residues and the glycan at position 386 has also been implicated in its binding site (3). However, this lectin did not show the same effects as GRFT suggesting that GRFT interacts with high-mannose glycans on gp120 somewhat differently to CV-N. Perhaps the arrangement of the 6 glycan binding sites on GRFT may allow greater cross-linking of glycans on gp120 compared to the 4 binding sites of CV-N. Recent structural studies on monomeric-GRFT have indicated that such intra-spike cross-linking is important for the antiviral activity of GRFT (42). This being said, it is clear that the GRFT enhancement is not due to cross-linking between viral particles as binding to the gp41 and anti-V3 mAbs was not enhanced. The competition between GRFT and 17b for binding to the HIV-2 virus 7312A in the presence of sCD4 suggested that GRFT may inhibit HIV-1 binding to the co-receptor after CD4 receptor engagement (Figure 6A and 6B). GRFT is likely to achieve this regardless of whether it binds before or after the CD4 receptor binding to gp120, although the effect is more pronounced when GRFT exposure precedes CD4 binding. However, the lack of steric obstruction between 17b and GRFT suggested that GRFT interference with the co-receptor binding site may not involve direct steric hindrance. We speculate that dimeric GRFT binding to multiple glycans on gp120 interferes with the structural re-arrangement induced by CD4 binding necessary to form the CD4i epitope. Lastly, the similarity in the potency of GRFT neutralization of CCR5 and CXCR4-mediated HIV-1 infection is likely to be related to the fact that this lectin inhibits the virus by targeting the viral envelope and not cellular receptors.

The CD4 binding site is a very important target for HIV-1 neutralizing antibodies since this site is conserved amongst different HIV-1 subtypes (14, 29, 61). This is supported by the fact that b12, one of the few HIV-1 broadly neutralizing antibodies targets this epitope (26). More recently highly potent anti-CD4bs mAbs, VRC01, VRC02 and VRC03 that neutralize a much broader spectrum of isolates from diverse genetic subtypes have been isolated (57). The vital role played by the CD4 receptor in the HIV-1 life cycle (14, 28) suggest that antibodies to this site could block replication *in vivo* as has been shown in macaques studies (49). Given the unique mode of action of lectins and the fact that most are not toxic *in vitro* or in animal models, it has been suggested these compounds represent a novel approach for intravenous targeting of HIV (2). As shown, the ability of GRFT to expose the CD4bs may offer a way of making HIV-1 more susceptible to neutralization by anti-CD4bs antibodies which are found in some HIV-positive patients (25). The study reported here increases our understanding of the interaction of GRFT with HIV-1 gp120 and also suggests a new way of increasing the exposure of the CD4bs.

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LEGENDS

Figure 1. HIV-1 capture by mAbs b12 and b6 was enhanced in the presence of GRFT. HIV-1 *env*-pseudotyped viruses from subtype B (QH0692.42 and PVO.4) and subtype C (COT6.15, COT9.6, Du151.2 and CAP239.G3J) were incubated with three different concentrations of GRFT before adding to plates coated with either b12 (A) or b6 (B). The amount of captured virus was assessed by p24 ELISA. Each mAb was also competed against itself as a positive control. The control (black bars) show the amount of virus captured on b12 or b6-coated plates in the absence of GRFT. The bars represent the mean ± SD of three different experiments.

Figure 2. CD4-IgG2 capture of HIV-1 was enhanced by GRFT. HIV-1 subtype B QH0692.42 (A) and subtype C COT6.15 (B) were incubated with different concentrations of GRFT and added to a plate coated with CD4-IgG2. The amount of captured virus was assessed by p24 ELISA relative to an untreated control with no GRFT (black bars). CD4-IgG2 was also competed against itself as a positive control. The bars represent the mean ± SD of three different experiments.

Figure 3. CV-N competed with b12 and b6 for binding to HIV-1. The subtype B pseudovirus QH0692.42 and subtype C pseudovirus COT6.15 were incubated with different concentrations of CV-N and then added to plates coated with either b12 (A-B) or b6 (C-D). The amount of captured virus was assessed by p24 ELISA relative to an untreated control with no CV-N (black bars). MAbs were also competed against themselves as positive controls. The bars represent the mean ± SD of three different experiments.

Figure 4. No enhancement of HIV-1 binding to F240, 4E10 and 3468L coated plates by GRFT. The subtype B viruse QH0692.42 was incubated with different concentrations of GRFT and then added to plates coated with the mAb F240 (A), 4E10 (B) and 3468L (C). The amount of captured virus was assessed by p24 ELISA relative to an untreated control with no GRFT (black bar). Each antibody was also competed against itself as a positive control. The bars represent the mean ± SD of three different experiments.

Figure 5. Effect of the 386 glycosylation site on the HIV-1 inhibition curves of GRFT and b12. (A) HIV-1 QH0692.42 and (B) COT6-V295N/S448N were neutralized in TZM-bl cells with GRFT and b12, in combination and alone. The shift in the inhibition curves of the two compounds combined (filled squares) relative to each compound alone (GRFT shown as filled triangles and b12 as open circles) are shown. (C) and (D) The same experiment described above but using QH0692-N386Q and COT6-V295N/S448N/N386Q, respectively. The dashed lines are placed at 50 and 80 percent inhibition. These graphs are representatives of three different experiments.

Figure 6. GRFT inhibition of the 17b mAb binding to the CD4i epitope. (A) The HIV-2 pseudovirus 7312A was first incubated with different concentrations of GRFT then with 25 μg/ml sCD4 prior to capture on a 17b-coated plate. The well containing the immobilized 17b only (0 μg/mL of GRFT and sCD4) is the experimental control well. The amount of captured virus was measured by p24 ELISA. Bars represent mean ± SD of three different experiments. (B) Same experiment as in (A) except that 7312A was first incubated with 25 μg/ml of sCD4 before the incubation with different concentrations of GRFT. (C) The HIV-2 pseudovirus 7312A was captured with CD4-IgG2 and then incubated with sCD4. This was followed by a sequential addition of 17b, GRFT and anti-GRFT antibody to the captured virus. The white bar (the well containing the virus and GRFT only) is the positive control while the black bar (the well containing the virus only) is the negative control. The amount of GRFT bound to the virus was measured by measuring the optical density at 450 nM after the addition of horse radish peroxidase and the substrate. Bars represent mean ± SD of three different experiments.

Figure 7. GRFT inhibited HIV-1 infection in U87-CCR5 and U87-CXCR4 cells. HIV-1 subtype C Du151, DS12, CM9 and RP1 were treated with different concentrations of GRFT before infection of U87-CCR5 (A) and U87-CXCR4 (B). Data are shown as the average plus standard deviations of three independent experiments. Untreated virus is shown in white (positive control). The IC_{50} values of GRFT are indicated next to each graph.

Table 1: Mannose-rich glycosylation pattern and GRFT mediated enhancement of HIV-1 binding to b12 and b6

HIV-1 envelope	Predicted N-linked mannose-rich glycosylation sites[a]											Fold increase with GRFT at 0.2 µg/mL	
pseudoviruses (subtype)	230	234	241	262	289	295	332	339	386	392	448	b12-coated plate	b6-coated plate
PVO.4 (B)	x									x		8.2 ± 2.1	11.1 ± 0.9
QH0692.42 (B)					x					x		4.1 ± 1.5	13.5 ± 1.7
COT6.15 (C)						x					x	4.5 ± 1.9	4.5 ± 1.8
Du151.2 (C)						x		x		x		2.3 ± 0.04	1.5 ± 0.2
CAP239.G3J (C)		x			x							1.5 ± 0.4	6.2 ± 1.2
COT9.6 (C)						x						1.5 ± 0.06	3.9 ± 2.1

[a] Mannose-rich glycosylation were identified from the amino acid sequence of each envelope clone (31).

Missing sites are marked by x. Viruses are ranked by subtype and according to their enhancement by GRFT.

Table 2: Effect of glycan mutations on GRFT-mediated enhancement of binding to b12 and b6

HIV-1 envelope	Predicted N-linked mannose-rich glycosylation sites [a]											Fold increase with GRFT at 0.2 µg/mL	
pseudoviruses	230	234	241	262	289	295	332	339	386	392	448	b12-coated plate	b6-coated plate
PVO.4	x									x		8.2 ± 2.1	11.1 ± 0.9
PVO-N386Q	x								x	x		4.8 ± 2.0	4.9 ± 1.9
QH0692.42					x					x		4.1 ± 1.5	13.5 ± 1.7
QH0692-N386Q					x				x	x		2.8 ± 0.3	6.5 ± 2.7
COT6.15						x					x	4.5 ± 1.9	4.5 ± 1.8
COT6-V295N/S448N												3.8 ± 0.02	8.7 ± 1.8
COT6-V295N/S448N/N386Q									x			1.7 ± 0.4	7.2 ± 2.8
COT9.6						x						1.5 ± 0.06	3.9 ± 2.1
COT9-V295N												2.8 ± 0.3	7.7 ± 3.2
COT9-V295N/N386Q									x			1.3 ± 0.2	1.7 ± 0.2

[a] Mannose-rich glycosylations were identified from the amino acid sequence of each envelope clone (31).
Missing mannose-rich glycosylation sites are marked by x.

Table 3: Synergy between GRFT and b12 for neutralization of HIV-1

HIV-1 envelope pseudoviruses	Single				Combined				CI [a]	
	GRFT		b12		GRFT		b12			
	IC_{50} [b] (μg/mL)	IC_{80} [c] (μg/mL)	IC_{50}	IC_{80}	IC_{50}	IC_{80}	IC_{50}	IC_{80}	IC_{50}	IC_{80}
QH0692.42	0.002	0.008	1.2	2.8	0.001	0.005	0.09	0.05	**0.6**	**0.6**
QH0692-N386Q	0.004	0.03	0.6	2.0	0.004	0.02	0.003	0.2	1.0	0.8
COT6.15	0.06	0.2	33.6	ND[d]	0.03	0.1	5.6	18.6	**0.7**	ND
COT6-V295N/S448N	0.003	0.008	44.1	ND	0.002	0.006	0.02	0.9	**0.7**	ND
COT6-V295N/S448N/N386Q	0.001	0.004	1.1	3.8	0.002	0.3	0.02	11.0	2.0	1.0
Du151.2	0.03	0.1	0.3	0.7	0.03	0.1	0.3	0.9	1.3	2.2
COT9.6	0.1	0.4	1.7	9.0	0.06	0.3	2.2	11.0	1.9	2.0

[a] The CI is the Combination Index: 0.3 to 0.7 indicates synergism, 0.7 to 0.85 indicates moderate synergism, 0.85 to 0.9 indicates slight synergism and 0.9 to 1.1 indicates an additive effect. Cases where synergism occurred are bolded.

[b] The IC_{50} is the concentration needed to reduce HIV-1 infection by 50%.

[c] The IC_{80} is the concentration needed to reduce HIV-1 infection by 80%.

[d] Not determined.

Table 4: Synergy between GRFT and HIV-1 positive plasma for neutralization of HIV-1

HIV-1 pseudovirus	BB10					BB34				
Envelope	Single		Combined		CI [a]	Single		Combined		CI
	GRFT	BB10	GRFT	BB10		GRFT	BB34	GRFT	BB34	
	IC_{50} [b] (nM)	ID_{50}	IC_{50} (nM)	ID_{50}		IC_{50} (nM)	ID_{50}	IC_{50} nM)	ID_{50}	
CAP239.G3J	0.3	0.009	0.06	0.0005	**0.2**	0.3	0.03	0.1	0.0008	**0.4**
COT6.15	1.4	0.03	0.7	0.005	**0.7**	1.4	0.0007	1.4	0.0007	1.1
COT9.6	0.7	0.01	0.3	0.003	**0.7**	0.6	0.03	0.3	0.02	1.2
QH0692.42	0.07	0.04	0.05	0.0003	**0.7**	0.1	0.009	0.2	0.002	2.2

[a] The CI is the Combination Index: 0.3 to 0.7 indicates synergism, 0.7 to 0.85 indicates moderate synergism, 0.85 to 0.9 indicates slight synergism and 0.9 to 1.1 indicates an additive effect. Cases where synergism occurred are bolded.

[b] The IC_{50} is the concentration needed to reduce HIV-1 infection by 50%.

Table 5: Synergy between GRFT and PRO140 for neutralization of HIV-1

HIV-1 envelope pseudoviruses	Single				Combined				CI [a]	
	GRFT		PRO140		GRFT		PRO140			
	IC_{50} [b] (nM)	IC_{80} [c] (nM)	IC_{50}	IC_{80}	IC_{50}	IC_{80}	IC_{50}	IC_{80}	IC_{50}	IC_{80}
PVO.4	0.1	0.1	9.4	33.5	0.1	0.1	2.3	5.1	1.2	1.2
QH0692.42	0.1	0.3	79.2	263.9	0.1	0.2	6.3	19.3	1.1	**0.7**
COT6.15	0.8	2.6	21.2	128.6	0.3	0.6	13.1	46.4	1.0	**0.6**
Du151.2	1.5	2.3	16.0	41.5	0.3	0.6	13.4	30.5	1.0	1.0
CAP239.G3J	0.2	0.9	36.2	165.8	0.2	0.4	6.5	22.1	1.2	**0.6**
COT9.6	2.7	6.3	31.0	147.2	0.5	1.6	23.8	53.0	1.0	**0.6**

[a] The CI is the Combination Index: 0.3 to 0.7 indicates synergism, 0.7 to 0.85 indicates moderate synergism, 0.85 to 0.9 indicates slight synergism and 0.9 to 1.1 indicates an additive effect. Cases where synergism occurred are bolded.

[b] The IC_{50} is the concentration needed to reduce HIV-1 infection by 50%.

[c] The IC_{80} is the concentration needed to reduce HIV-1 infection by 80%.

Figure 1A

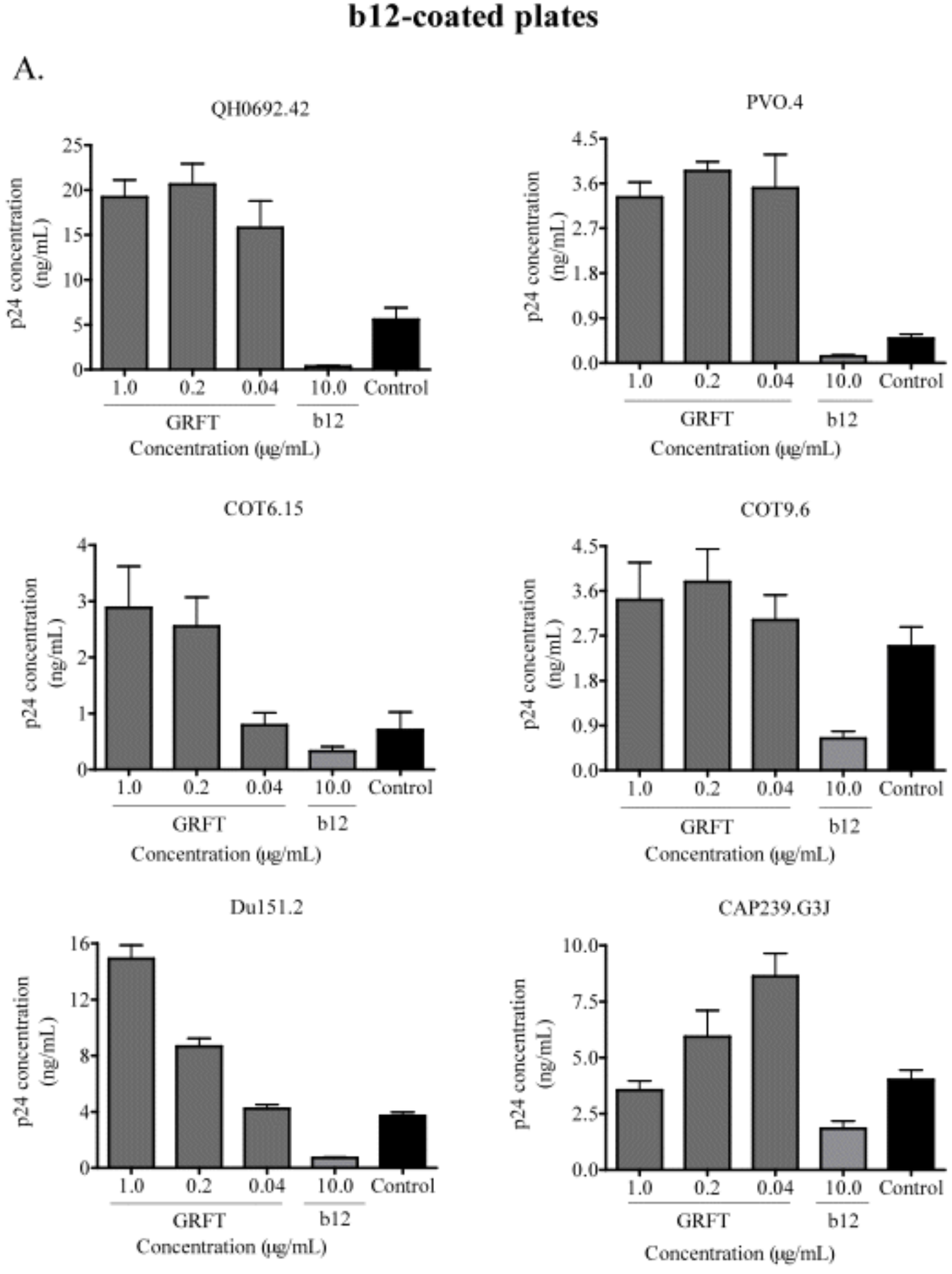

b12-coated plates
A.
QH0692.42
PVO.4
COT6.15
COT9.6
Du151.2
CAP239.G3J
p24 concentration (ng/mL)
1.0
0.2
0.04
10.0
Control
GRFT
b12
Concentration (µg/mL)

Figure 1B

b6-coated plates

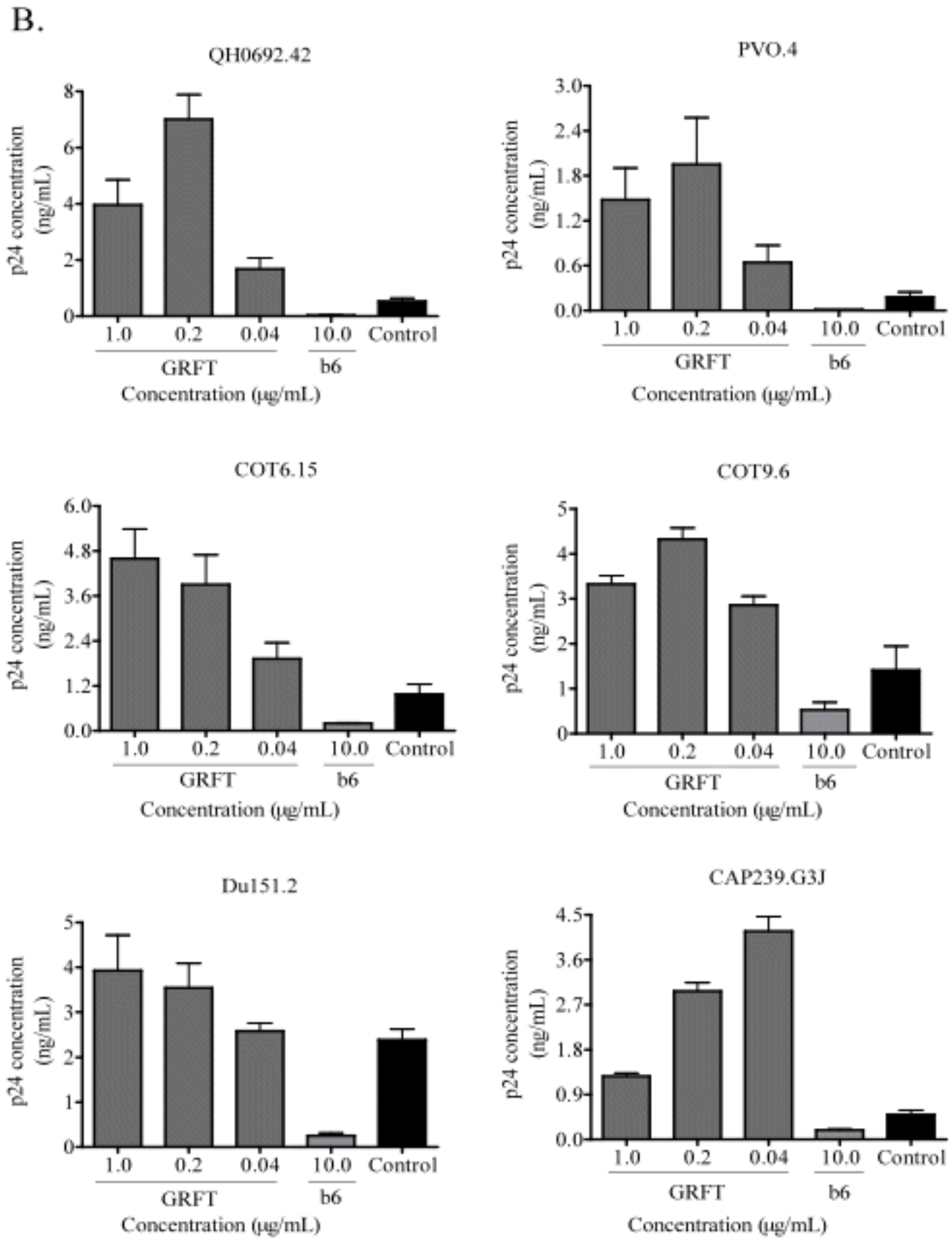


Figure 2

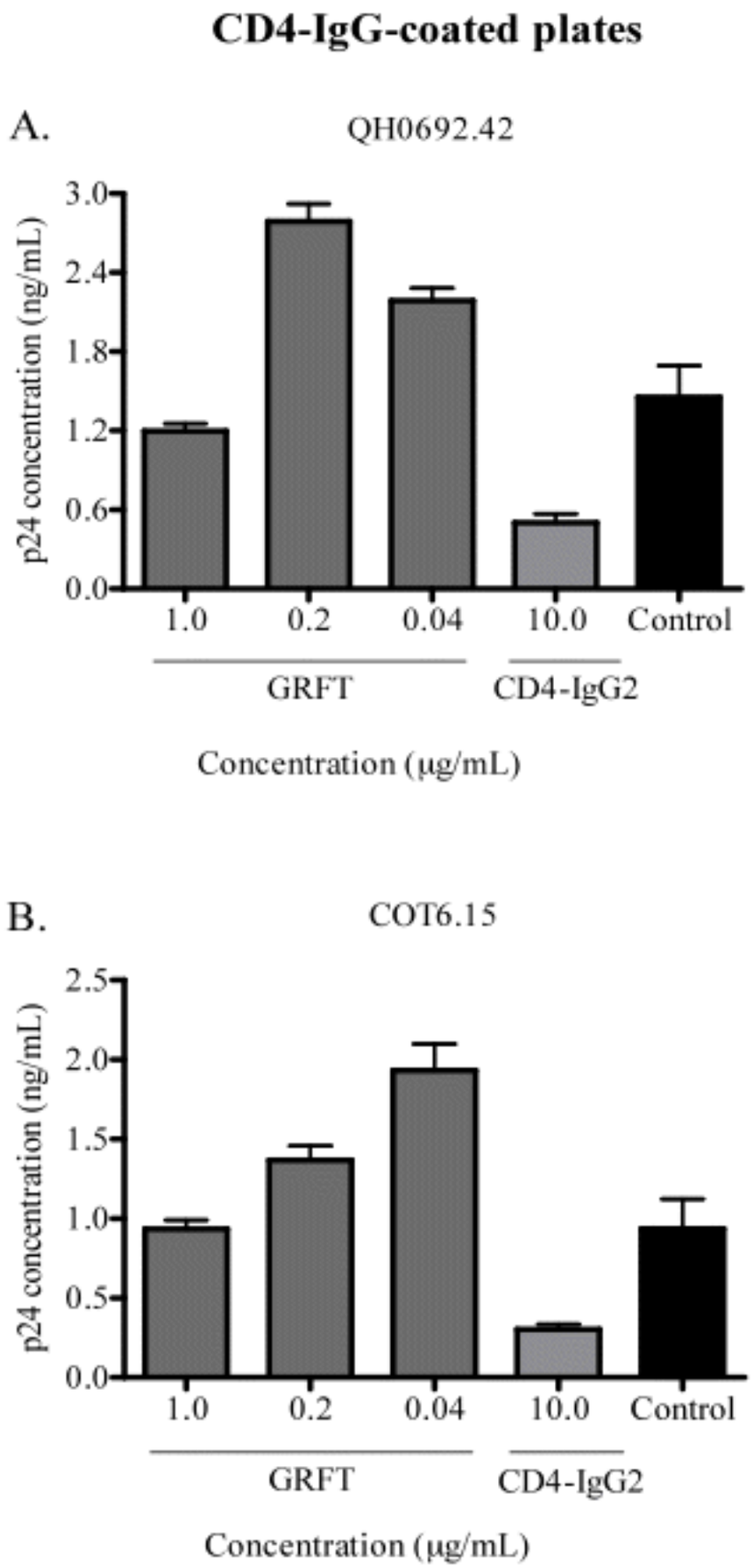

CD4-IgG-coated plates
A.
QH0692.42
p24 concentration (ng/mL)
3.0
2.4
1.8
1.2
0.6
0.0
1.0
0.2
0.04
10.0
Control
GRFT
CD4-IgG2
Concentration (µg/mL)
B.
COT6.15
p24 concentration (ng/mL)
2.5
2.0
1.5
1.0
0.5
0.0
1.0
0.2
0.04
10.0
Control
GRFT
CD4-IgG2
Concentration (µg/mL)

Figure 3

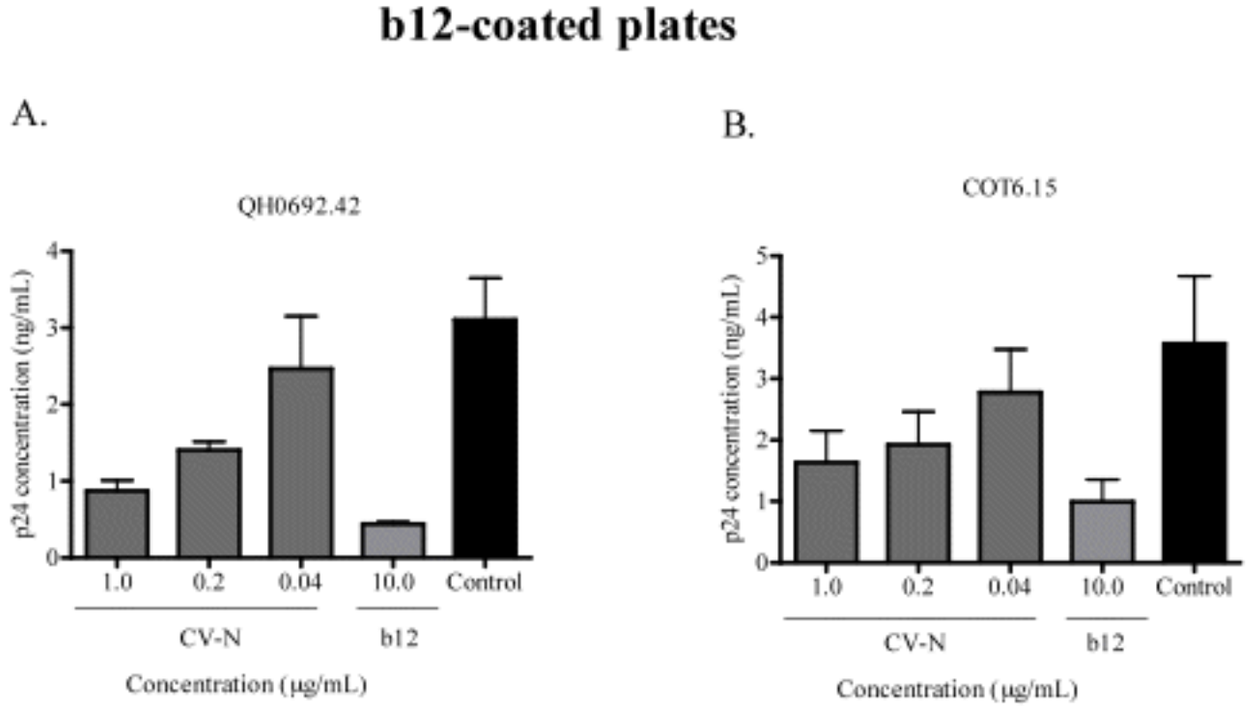
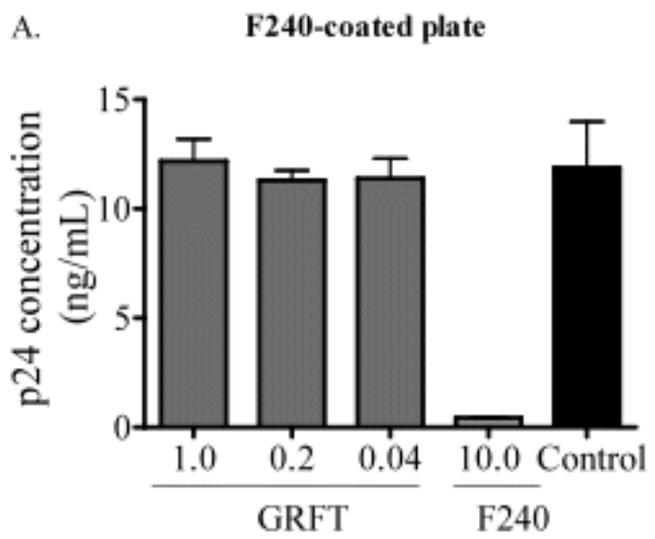
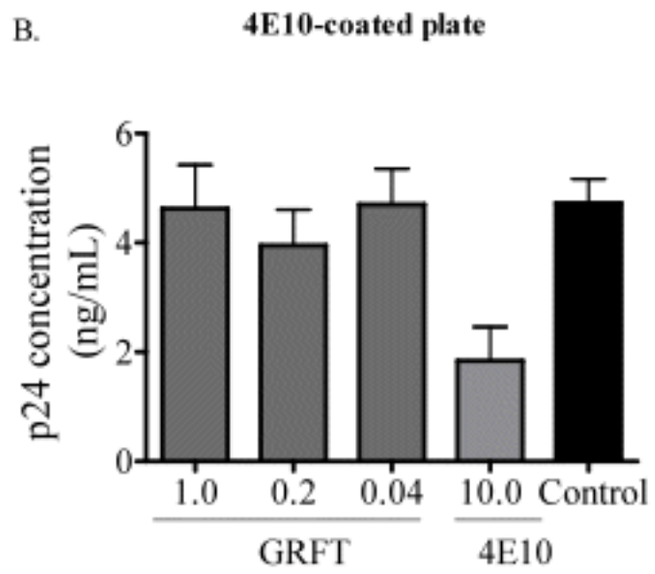

b12-coated plates
A.
QH0692.42
p24 concentration (ng/mL)
0
1
2
3
4
1.0
0.2
0.04
10.0
Control
CV-N
b12
Concentration (µg/mL)
B.
COT6.15
p24 concentration (ng/mL)
0
1
2
3
4
5
1.0
0.2
0.04
10.0
Control
CV-N
b12
Concentration (µg/mL)
b6-coated plates
C.
QH0692.42
p24 concentration (ng/mL)
0
1
2
3
4
1.0
0.2
0.04
10.0
Control
CV-N
b6
Concentration (µg/mL)
D.
COT6.15
p24 concentration (ng/mL)
0
1
2
3
4
1.0
0.2
0.04
10.0
Control
CV-N
b6
Concentration (µg/mL)

Figure 4


A.
F240-coated plate
p24 concentration (ng/mL)
15
10
5
0
1.0
0.2
0.04
10.0
Control
GRFT
F240


B.
4E10-coated plate
p24 concentration (ng/mL)
6
4
2
0
1.0
0.2
0.04
10.0
Control
GRFT
4E10

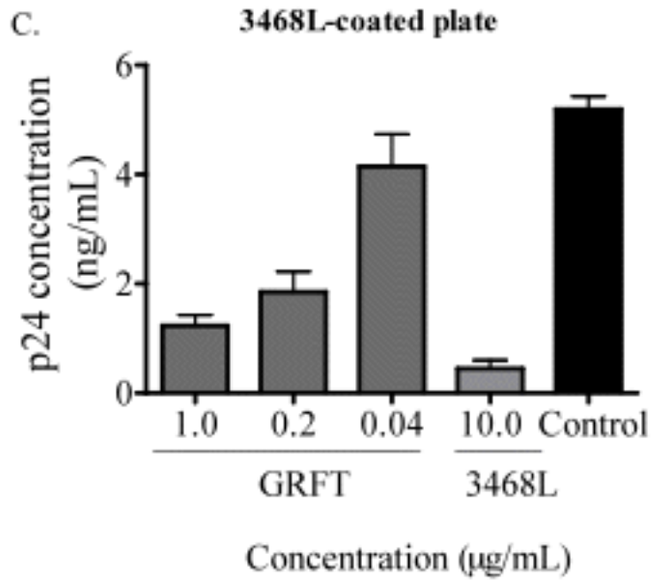

C.
3468L-coated plate
p24 concentration (ng/mL)
6
4
2
0
1.0
0.2
0.04
10.0
Control
GRFT
3468L
Concentration (µg/mL)

Figure 5

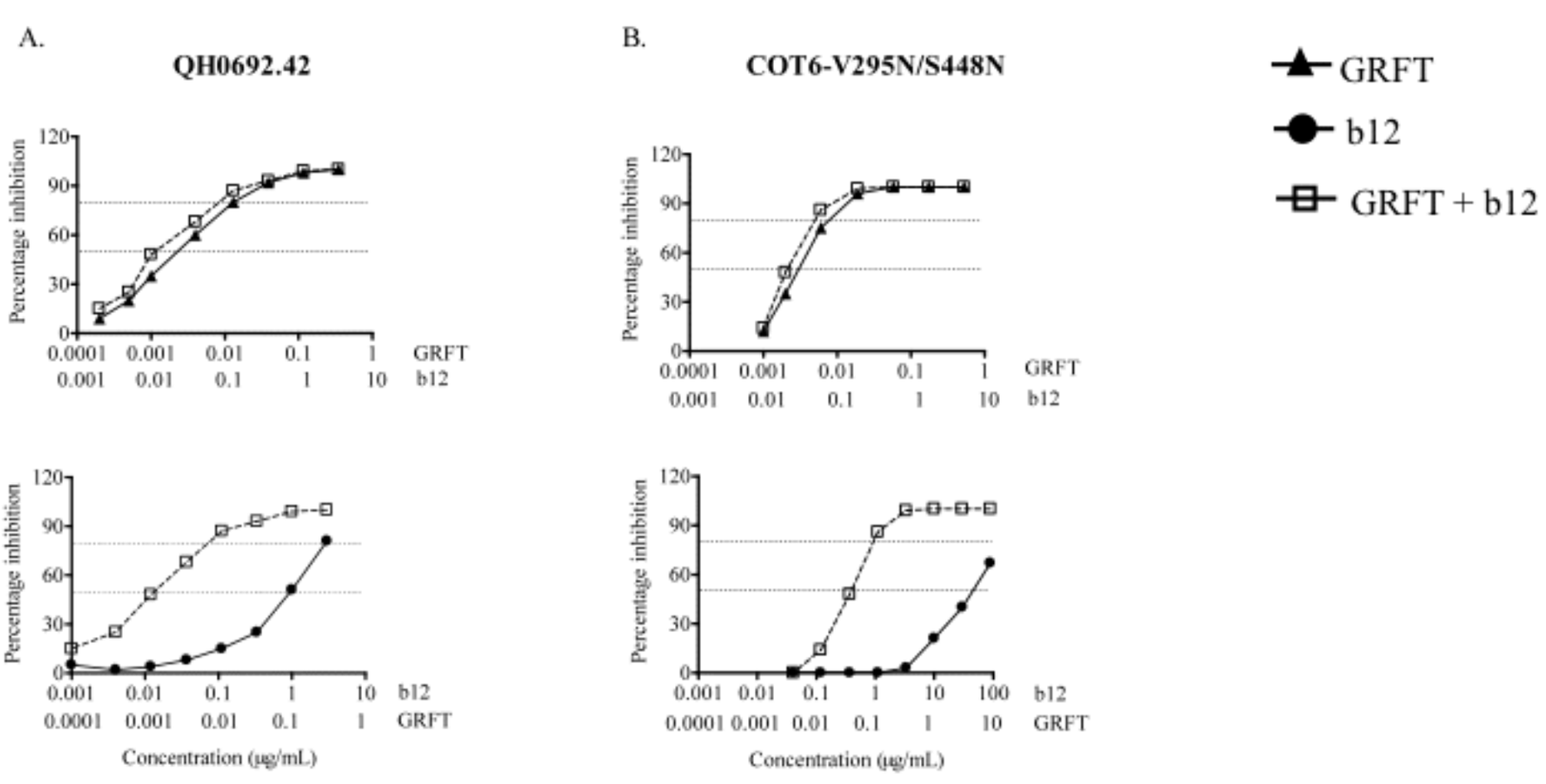
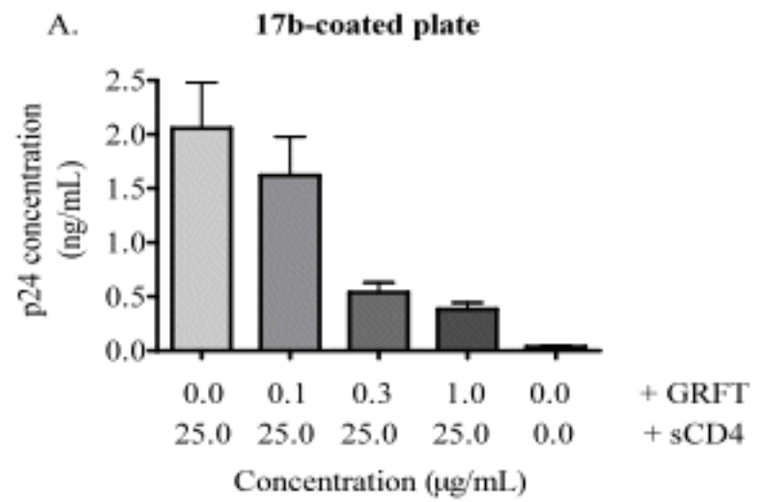
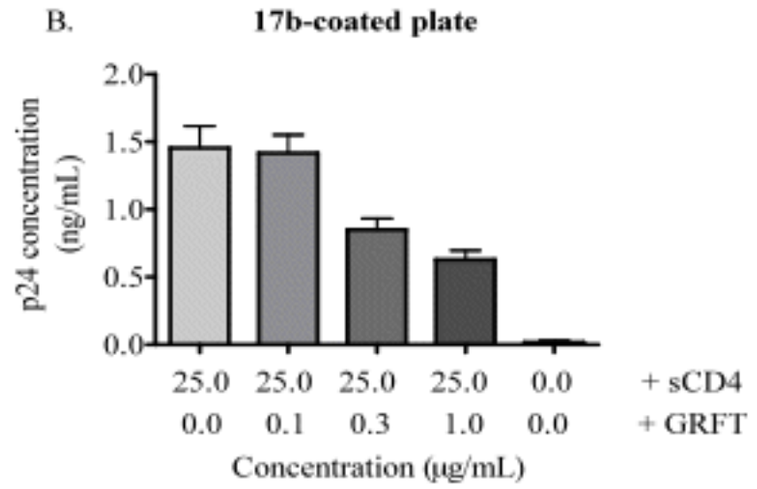

A.
QH0692.42
B.
COT6-V295N/S448N
GRFT
b12
GRFT + b12
Percentage inhibition
120
90
60
30
0
0.0001 0.001 0.01 0.1 1 GRFT
0.001 0.01 0.1 1 10 b12
0.001 0.01 0.1 1 10 b12
0.0001 0.001 0.01 0.1 1 GRFT
0.001 0.01 0.1 1 10 100 b12
0.0001 0.001 0.01 0.1 1 10 GRFT
Concentration (μg/mL)
C.
QH0692-N386Q
D.
COT6-V295N/S448N/N386Q

Figure 6


A.
17b-coated plate
p24 concentration (ng/mL)
2.5
2.0
1.5
1.0
0.5
0.0
0.0 0.1 0.3 1.0 0.0 + GRFT
25.0 25.0 25.0 25.0 0.0 + sCD4
Concentration (µg/mL)


B.
17b-coated plate
p24 concentration (ng/mL)
2.0
1.5
1.0
0.5
0.0
25.0 25.0 25.0 25.0 0.0 + sCD4
0.0 0.1 0.3 1.0 0.0 + GRFT
Concentration (µg/mL)

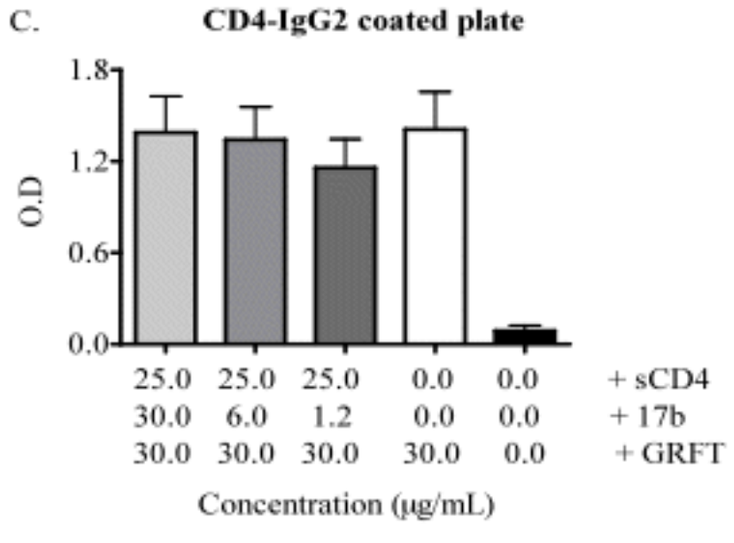
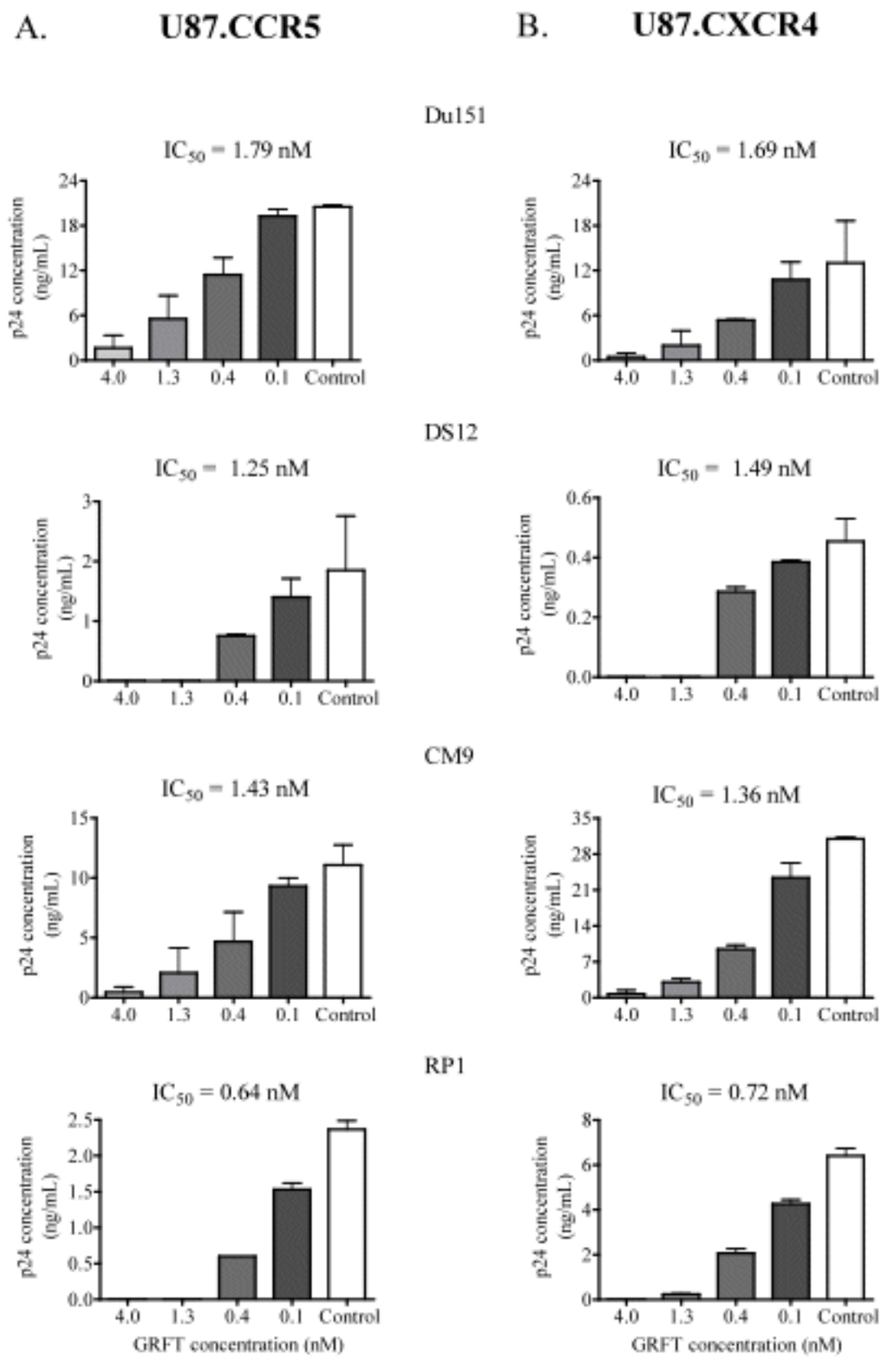

C.
CD4-IgG2 coated plate
O.D
1.8
1.2
0.6
0.0
25.0 25.0 25.0 0.0 0.0 + sCD4
30.0 6.0 1.2 0.0 0.0 + 17b
30.0 30.0 30.0 30.0 0.0 + GRFT
Concentration (µg/mL)

Figure 7


A.
U87.CCR5
B.
U87.CXCR4
Du151
IC50 = 1.79 nM
IC50 = 1.69 nM
DS12
IC50 = 1.25 nM
IC50 = 1.49 nM
CM9
IC50 = 1.43 nM
IC50 = 1.36 nM
RP1
IC50 = 0.64 nM
IC50 = 0.72 nM
p24 concentration (ng/mL)
4.0
1.3
0.4
0.1
Control
GRFT concentration (nM)