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(57) Abstract: The present invention relates, in general, to HIV and, in particular, to novel immunogens suitable for use in inducing an immune response to HIV.



IMMUNOGENS

This application claims priority from U.S. Provisional Application No. 61/473,748, filed April 9, 2011, the entire content of which is incorporated herein by reference.

5 This invention was made with government support under Grant No. CHAVI U19 AI067854 awarded by the National Institutes of Health. The government has certain rights in the invention.

TECHNICAL FIELD

10 The present invention relates, in general, to HIV and, in particular, to novel immunogens suitable for use in inducing an immune response to HIV.

BACKGROUND

Antibodies that bind to the Fcγ-Receptor (FcR) IIIa on the surface of natural killer (NK) cells can mediate antibody dependent cytotoxic activity (ADCC) or antibody dependent cellular viral inhibition (ADCVI) (4). In addition, 15 binding of immunoglobulin (Ig) Fc to FcR can induce anti-HIV-1 chemokine release (5, 29). These types of effector functions have been implicated in protective immune responses against HIV-1(3, 12, 20). Several studies have reported active and passive immunization that protected from SIV or SHIV infection in non-human primates. The mechanism of protection was related at 20 least in part, to ADCC- and ADCVI-mediating antibodies (11, 13, 16, 17, 35). Antibodies that mediate FcR-dependent anti-HIV-1 activities that are non-neutralizing in conventional HIV-1 neutralizing assays have been postulated to be a correlate of protection in the Thai RV1144 gp120 vaccine efficacy trial (15, 26).

25 FcR-mediated antibody activity is dependent on both the state of glycosylation of the Fc region (2, 23, 25), as well as on the specificity of the Fab

region (i.e. the antibody must target epitopes on the surface of virus-infected cells). While the epitopes involved in mediating virus neutralization have been comprehensibly profiled, HIV-1 epitopes that are capable of mediating ADCC and ADCVI in HIV-1 infection have not been adequately studied. Thus, an analysis has been undertaken of existing non-neutralizing anti-Env human monoclonal antibodies (mAbs) for their ability to bind to HIV-1 infected cells and to sensitize target CD4+ T cells for ADCC.

A human mAb (A32) recognizes a conformational epitope involving the C1 and C4 gp120 regions following Env binding to CD4 (22). The present invention results, least in part, from a study demonstrating that the A32 epitope is expressed on the surface of transmitted/founder virus -infected CD4 T cells beginning at day 3 of *in vitro* infection, and can mediate potent ADCC activity with both virus-infected and gp120-coated CD4 T cells. Moreover, mAb A32 Fab blocks the majority of ADCC antibody activity in HIV-1 chronically infected sera.

SUMMARY OF THE INVENTION

The present invention relates, in general, to HIV and, in particular, to novel immunogens suitable for use in inducing an immune response to HIV.

Objects and advantages of the present invention will be clear from the description that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Detection of Env expression on the cellular membrane of the A1953_SupT1 cell line. A1953-SupT1 cells ($0.1-0.2 \times 10^6$) were stained with five different concentrations of primary Ab as indicated in the figure (range 20-0.5 $\mu\text{g/ml}$). The primary Ab preparations were: 1) mAb A32 and Synagis®

(negative control); 2) IgG preparations HIVIG and IVIG as positive and negative controls, respectively. The results are reported overlaying histograms representing the cells after incubation with no Ab (unstained; gray area), with the secondary Ab alone (Secondary Ab Alone; black curve), and with primary and secondary Ab (primary Ab + secondary Ab; green curve). Where positive staining was detected, the Median Fluorescence Intensity (MFI) of the positive population is reported.

Figure 2. Detection of Env expression on the cellular membrane of HIV-infected CD4⁺ T cells. Activated CD4⁺ T cells were infected with two HIV-1 strains, the laboratory adapted NL4-3 and transmitted/founder primary CH077.t infectious molecular clones, as described in Example 1 below. Replicate cultures of mock and HIV-infected cells were plated in a 96 well/plate. Each cell condition was harvested starting 3 days post-infection and every 24 hours thereafter to detect the expression of Env proteins on the cellular membrane. One-two hundred thousand cells were stained with 10µg/ml of the mAb A32, 17b, and 2G12 as well as with the IgG preparation HIVIG as primary Ab source. The Synagis® and IVIG were used as negative controls for the mAb and IgG preparation, respectively. The results are reported overlaying histograms representing the cells after incubation with no Ab (unstained; gray area), with the secondary Ab alone (Secondary Ab Alone; black curve), and with primary and secondary Ab (primary Ab+ secondary Ab; green curve).

Figures 3A-3C. Antibody Dependent Cellular Cytotoxicity (ADCC) activity. The ability of mAbs A32 (red line), 17b (green line), and 2G12 (blue line) to mediate ADCC activity was tested using gp120_{WITO}-coated (Fig. 3A), gp140_{CH040} (Fig. 3B), and A1953-chronically infected (Fig. 3C) CEM.NKR_{CCR5} cell line. The Synagis® was used as negative control (black line). The mAb were tested starting

at a concentration of 40 µg/ml using 4-fold dilutions. The results are reported as %GzB activity after background was subtracted. The results represent the average of triplicate experiments ± standard deviation. The black dotted line represents the cut-off (8%GzB activity). PBMC from a healthy HIV-1 seronegative donor were
5 used as source of effector cells and for an effector to target cell ratio of 30:1 in all experiments.

Figure 4. Antibody Dependent Cellular Cytotoxicity (ADCC) activity. The ability of mAbs A32, HIVIG (positive control) and Synagis® (negative control) to mediate ADCC activity was tested at the concentration indicated on the axis.
10 Activated PB CD4+ T cells from a healthy HIV-1 seronegative donor after 3-day infection with the IMC, NL-LucR.T2A-BaL.ecto, were used as target cells. NK cells obtained by negative selection from autologous PBMC were used as source of effector cells. The effector to target cell ratios of 40 (red bars) and 10 (blue bar) to 1 were used. The results are reported as %GzB activity after background was
15 subtracted.

Figures 5A and 5B. Inhibition of ADCC activity by A32 Fab fragment. The A1953_CEM.NKR_{CCR5} cell line as used as target cells and PBMC from a healthy HIV-1 seronegative donor as source of effector cells. The effector to target cell ratio was 30:1 in all experiments. The results are reported as %GzB activity after
20 background was subtracted. The values above the bars represent the percentage of inhibition when the A32 Fab fragment was used in the pre-incubation. Fig. 5A. The target cells were pre-incubated with medium only (pre-treatment, red bars), with 10 µg/ml of the A32 Fab fragment (blue bars), or with 10 µg/ml of the Synagis® (black bars). The results represent the average of results from triplicate
25 experiments. After washing, the cells were incubated with the mAb A32 at 10

µg/ml. Fig. 5B. The target cells were pre-incubated as previously described with medium only, A32 Fab or Synagis®. After washing, the plasma collected from each donor were subsequently added at the dilutions indicated in parenthesis. The mAb A32 and HIVIG preparation were used as positive controls.

- 5 Figure 6. Shown is the sequence of the HIV-1 glycoprotein 120 (gp120). The binding site for the monoclonal antibodies (mAb) A32 is highlighted. The monoclonal antibodies are capable of binding a conformational site on the gp120 that includes C1 and C2 regions.

- Figure 7. As shown in Fig. 5A, if the target cells used in the assay were pre-
10 incubated treated with the A32-Fab, the A32 mAb could not bind to the gp120 and mediate ADCC. The same applies to the ADCC activity mediated by the Ab present in the plasma collected from HIV-1 infected individuals. The HIV-1 A1953 chronically infected cell line was used as target cells without pre-incubation (red dots) or incubated with the A32-Fab (blue dots) or the control Ab
15 (black dot). Pre-incubation with A32-Fab but not with control Ab was able to reduce the ADCC activity mediated by Ab present in the plasma of different groups of HIV-1 infected individuals. Each dot represents one individual and the lines connect the dots representing the same individual in each of the experimental conditions. The results are reported as percentage of Granzyme B
20 (%GzB) activity after background was subtracted.

Figure 8. The same methodology was applied to evaluate the presence of A32-like Ab responses capable of mediating ADCC activity in RV144 vaccine recipients. The effect of pre-incubation with the A32-Fab was even more dramatic in this group with a significant decrease in the %GzB activity when the plasma

was added to target cells pre-incubated with A32-Fab compared to that observed when no pre-incubation was performed.

Figure 9. Specificity of RV144 induced antibodies.

Figure 10. RV144 induced antibody responses.

- 5 Figure 11. Quaternary mAb CHO1 binding to A244 gp120 is abrogated by N160K mutation.

Figure 12. Differential binding of RV144 vaccinee purified IgG to A244WT versus A244 N160K gp120.

DETAILED DESCRIPTION OF THE INVENTION

- 10 Among non-neutralizing HIV-1 envelope antibodies, those capable of mediating antibody dependent cellular cytotoxicity (ADCC) activity have been postulated to be important for control of HIV-1 infection. ADCC-mediating Ab must recognize HIV-1 antigens expressed on the membrane of infected cells and bind the Fc γ -receptor (Fc γ -R) of the effector cell population. However, the precise
15 targets of serum ADCC antibody are poorly characterized. The human mAb A32 is a non-neutralizing antibody isolated from a HIV-1 chronically infected person. An investigation was made of the ability of mAb A32 to recognize HIV-1 envelope expressed on the surface of CD4⁺ T cells infected with primary and laboratory adapted strains of HIV-1, as well as of its ability to mediate ADCC
20 activity. Mab A32 epitope was expressed on the surface of HIV-1 infected CD4⁺ T cells earlier than the CD4-inducible (CD4i) epitope bound by mAb 17b and the

gp120 carbohydrate epitope bound by mAb 2G12. Importantly, mAb A32 was a potent mediator of ADCC. Finally, an A32 Fab blocked the majority of ADCC-mediated Ab activity in chronic HIV-1 infection serum. These data demonstrate that the epitope defined by mAb A32 is a major target on gp120 for plasma
5 ADCC activity.

Previous studies have demonstrated the frequent induction of CD4i antibody responses in acute (27) and chronic (7) HIV-1 subtype B infection and that they contribute to the neutralizing activity in acute subtype C infection (14). These data, together with the observations of the ability of mAb A32 to recognize
10 Env expression on the surface of HIV-1-infected cells earlier than 17b and 2G12, emphasizes the importance of the A32 epitope as a candidate for the target of non-neutralizing gp120 antibodies that may participate in protection for HIV-1 acquisition. The present studies also demonstrate the importance of understanding what role A32-like antibodies may have in preventing HIV-1 acquisition in
15 vaccine trials such as RV144 (26).

The present invention relates to HIV envelope immunogens that induce the production in a subject (e.g., a human) of A32-like antibodies, that is, antibodies that are blocked by A32 Fab binding. The invention further relates to nucleic acids encoding such antigens and to vectors comprising same. The
20 invention also relates to methods of inducing an immune response against HIV in a subject(e.g., a human) by administering an effective amount of such immunogens/encoding nucleic acids.

Certain aspects of the invention can be described in greater detail in the non-limiting Example that follows. (See also U.S. Provisional Application
25 No. 61/407,299, filed October 27, 2010.)

EXAMPLE 1

Experimental Details

Monoclonal antibodies and HIVIG preparation. The A32 and 17b monoclonal antibodies (mAb) utilized in this study were originally isolated by James Robinson (Tulane University, New Orleans, LA). The 2G12 mAb was purchased from Polymun (POLYMUN Scientific Immunobiologische Forschung GmbH, Vienna, Austria). The humanized monoclonal antibody (IgG1_k) directed to an epitope in the A antigenic site of the F protein of respiratory syncytial virus, Synagis® (palivizumab; MedImmune, LLC; Gaithersburg, MD) was purchased from the manufacturer and used as a control. Human polyclonal anti-HIV-1 IgG preparation was used as a positive control from NIH AIDS Research & Reference Reagent Repository (HIVIG lot#114; (6)).

Target cells. CEM.NKR_{CCR5} cell line (31), chronically infected SupT1 CD4⁺ T cells, and activated peripheral blood (PB) CD4⁺ T cells were used as target cells. PB CD4⁺ T cells were generated from cryopreserved PB Mononuclear Cells (PBMC) as follows. PBMC samples obtained from an HIV-seronegative donor were thawed and activated by 72-hour culture at 37°C and 5% CO₂ in RPMI 1640 medium (Invitrogen, Carlsbad, CA), supplemented with 20% Fetal Bovine Serum (FBS) (Gemini Bio-Products, West Sacramento, CA) (R20) in presence of 20 U/ml rhIL-2 (Peprotech, Rocky Hill, NJ) (R20-IL2), and containing anti-CD3 (clone OKT3, eBioscience, San Diego, CA) and anti-CD28 (clone CD28.2, BD Biosciences, San Jose, CA) at 150 ng/ml. After activation, the CD4⁺ enriched population was obtained by removing CD8⁺ T cells by magnetic bead separation (Miltenyi Biotec). All the samples from the seronegative donors were obtained according to institutional IRB protocol.

Recombinant gp120 HIV-1 proteins. The recombinant gp120 HIV-1 protein from the WITO early-infection env-clone (Genebank No. AY835451; Immune Technology Corp, New York) and the recombinant gp140 cleavage-site deficient (C) oligomer from the transmitted/founder recombinant Env from subject CH040 (18). For each recombinant gp120 or gp140, the optimum amount

to coat target cells was determined as that amount able to compete $\geq 50\%$ the binding of the anti-CD4 Leu3A mAb antibody (BD Bioscience, San Jose, CA, USA). This competition was determined as reduction of the mean fluorescence intensity of Leu 3A reactivity in gp120-coated CEM.NKR_{CCR5} cells compared to control cells.

Virus, infectious molecular clones (IMC), chronically infected cells. The following three replication-competent infectious molecular clones (IMC) of clade B HIV-1 strains were used: X4-tropic pNL4-3 (Genebank No. M19921 & AF324493.2)(1); R5-tropic NL-LucR.T2A-BaL.ecto which expresses BaL env sequence (Genebank No. AY426110) in cis in an isogenic, pNL4-3 based reporter virus backbone that preserves all viral orfs as described by Edmonds et al. (8); and recently generated full-length IMC, pCH077.t (Ochsenbauer et al., manuscript in preparation), representing the transmitted / founder virus strain from subject CH077 (28). Virus stocks were generated by transfection of 293T cells with proviral DNA, and titered on TZMbl cells. The A1953 virus was derived from the HXB2 isolate selected for the inability to downregulate CD4 from the surface of chronically infected T-cell lines. It was subsequently shown to be defective in Nef expression. SupT1 and CEM.NKR_{CCR5} cells that are chronically infected with A1953 characteristically express high levels of surface CD4 bound to gp120 (i.e., detectable with OKT4, which does not compete with gp120 for binding to CD4; and not detectable by anti-CD4 antibodies known to compete with gp120). These A1953-infected cells express high levels of CD4 complexed with envelope glycoprotein on the cell surface (J. Hoxie, unpublished data). Chronically infected A1953_SupT1_{CCR5} and A1953_CEM.NKR_{CCR5} cell lines were used to evaluate the binding capacity and ADCC activity of A32 mAb to the env protein expressed on the surface of HIV-1 infected cells, respectively.

Effector cells. PBMC obtained by leukopheresis from a seronegative donor were cryopreserved and subsequently used as effector cell population or as

the source of purified natural killer (NK) cells. Cryopreserved PBMC were thawed, and then rested overnight at 2×10^6 cell in in RPMI1640 medium (Invitrogen) supplemented with 10% FBS (R10). Cells were counted for viability and adjusted to the proper concentration to obtain an effector to target (E:T) ratio of 30:1. In experiments where infected $CD4^+$ -enriched cells were used as targets, NK cell populations were isolated from cryopreserved PBMCs by negative selection with magnetic beads (Miltenyi Biotec). In these experiments, NK effector cells were used at an E:T ratio of 10:1.

Indirect surface immunofluorescence analysis of HIV-1-infected $CD4^+$ T cells. Activated $CD4^+$ -enriched T cells and A1953_SupT1 cells were obtained as described above. $CD4^+$ T cells were infected by spinoculation ($1200 \times g$ for 2 hours; (24)) with 0.33 TCID₅₀/cell of HIV-1 virus stock derived from the IMCs, pNL4-3 (accession number M19921 & AF324493.2)(1) and pCH077.t (Ochsenbauer et al., manuscript in preparation), respectively, representing a X4-tropic reference strain of HIV-1, and the transmitted/founder virus from subject CH077 (28). Cells spinoculated in the absence of virus (mock-infected) were used as a negative infection control. Following 72 hours of infection in R20-IL-2, $CD4^+$ -enriched T cells were washed in PBS, dispensed in 96-well V-bottom plates at 1×10^5 viable cells/well, and stained with a vital dye (LIVE/DEAD Fixable Aqua Dead Cell Stain, Invitrogen) to exclude non-viable cells from subsequent analyses. The cells were then washed twice with 250 μ l/well of washing buffer (WB; PBS+1% FBS), and incubated at 4°C for 25 minutes with the primary Ab or IgG preparation. After two washes, cells were stained with Phycoerythrin (PE)-conjugated goat anti-human IgG secondary (2ary) Ab (Southern Biotech, Birmingham, AL) for 25 minutes at 4°C. Cells were subsequently washed 3 times and fixed with 1% formaldehyde PBS. The samples were acquired on a LSRII (BD Biosciences) within 24 hours. A minimum of 10,000 total singlet events was

acquired for each test to identify live events. Data analysis was performed using FlowJo 8.8.4 software (TreeStar Inc., Ashland, OR).

Longitudinal Env Expression Assay. To assess the time course of env antigen expression on the membrane of CD4⁺-enriched T cells, PBMC were
5 stimulated and cultured at a concentration of 1×10^6 cells/mL in RPMI +20% FBS supplemented with 0.05mg/mL Gentamicin (Sigma), 20U/mL recombinant human IL-2 (Peprotech) (R20-IL2), 50ng/mL OK-T3 (R&D Systems), and 100ng/mL α -CD28 (BD Biosciences). These cultures were incubated at 37°C and 5% CO₂ for 72 hours. CD8⁺ T-cells were depleted from 1.5×10^9 stimulated
10 hPBMCs via Miltenyi separation (Miltenyi Human Microbead Kit) using the AUTOMacs Deplete/Rinse program. CD4⁺-enriched T cells were then resuspended at 10×10^6 cells/mL in R20-IL2 and bulk infected with 0.977 TCID₅₀/cell of CH077.t virus stock via spin-inoculation at 1200 RCF for 2 hours. Virus infected cells were then split into the appropriate number of flasks for
15 culture and incubated at 37°C and 5% CO₂ for 3-7 days. Days 3-7 post-infection, viral envelope expression was analyzed by indirect immunofluorescence staining of virus infected CD4⁺-enriched T cells (1×10^6 cells) with the appropriate antibodies at a final concentration of 10ug/mL. Additionally, culture supernatants were collected and virus infectivity was analyzed via TZM-bl assay.

20 *ADCC assay.* Antibody Dependent Cellular Cytotoxic (ADCC) activity was detected according to modifications of the previously described GranToxiLux (GTL) procedure (19). Infected and uninfected target cells in 96 well plates were counted, washed, resuspended in RPMI1640 medium (Invitrogen) supplemented with 10% FBS (R10) at 1×10^6 cell/ml, and labeled with a fluorescent target-cell
25 marker (TFL4; OncoImmunin, Inc., Gaithersburg, MD) and a viability marker (NFL1; OncoImmunin, Inc.) for 15 minutes in a 37°C water bath, followed by two washes. Both effector and target populations were counted and concentrations were adjusted to reach a final effector to target ratio of 30:1 and 10:1 when

PBMC and NK cells were used respectively. For the A32-Fab blocking assay, the A1953_CEN.NKR_{CCRS} target cells were plated, incubated for 15 min at 37°C and washed once before the effector cells were added to the wells. GzB substrate (OncoImmunin, Inc.) was dispensed into each well of a 96-well V-bottom plate to reach a final GzB substrate concentration of 0.25x. After incubation for 5 minutes at room temperature (RT), 25µl of the appropriate antibody and IgG dilutions were added to the target/effector cell suspension and then incubated for 15 minutes at RT. The plates were centrifuged for 1 minute at 300xg, and incubated for 1 hour at 37°C and 5% CO₂. After two washes with WB, cells were resuspended in 225µl of WB, placed at 4°C, and acquired with the LSRII within 5 hours. A minimum of 5 and 10x10³ events representing viable gp120-coated and infected target cells, respectively, was acquired for each well. Data analysis was performed using FlowJo 8.8.4 software. The results are expressed as %GzB activity, defined as the percentage of cells positive for proteolytically active GzB out of the total viable target cell population. The final results are expressed after subtracting the background represented by the %GzB activity observed in wells containing effector and target cell populations in absence of mAb, IgG preparation, or plasma. The results were considered positive if %GzB activity after background subtraction was >8% for the gp120-coated and A1953 target cells. For the IMC-infected target cells, the results were considered positive if %GzB activity after background subtraction was >15%.

Results

Binding of mAb A32 to the surface of HIV-1 A1953-chronically infected SupT1 cell line. The first question asked was whether the A32 epitope was expressed on the surface of HIV-1 infected CD4⁺ T cells. Observed were saturation binding levels of A32 mAb between 5-20 µg/ml and peak of mean fluorescence intensity at 10µg/ml on the A1953-infected SupT1 CD4⁺ T cell line.

Most of the A1953-infected SupT1 CD4⁺ T cells were positive for A32 mAb reactivity (Figure 1).

Expression of A32 binding site on the surface of HIV-1 infected PB CD4⁺ T cells. CD8-depleted PBMCs obtained from HIV-1 seronegative healthy subjects
5 were activated for three days using the anti-CD3/CD28 Ab combination (9) and infected with either an infectious molecular clone (IMC) of the transmitted/founder virus CH077.t or the laboratory-adapted HIV-1 strain, NL4-3 at a multiplicity of infection of 0.33 TCID₅₀/cell. Mock-infected CD8-depleted cells from the same donor were used as uninfected cell controls. Cells were
10 harvested and mAb surface reactivity determined at 24 hours intervals starting 72 hours post-infection and continuing through day 7. The A32, 17b, 2G12 mAbs and controls were used at 10µg/ml saturating concentration (Figure 2). Neither the three mAbs nor the HIVIG preparation nor the negative controls (Synagis® and IVIG) bound to mock-infected cells. In contrast, the expression of Env antigens
15 on HIV-1-infected cells was always detected with A32 mAb on day 3 of infection before expression of both CCR5 binding site (17b mAb) and the gp120 carbohydrate epitope defined by 2G12 mAb, and also before epitopes recognized by HIVIG. MAbA32-reactive CD4⁺ T cells infected with the R5 transmitted/founder IMC, CH077.t appeared on day 3 of *in vitro* infection whereas PB CD4⁺
20 T cells infected with NL4-3 X4 HIV-1 appeared on day 5. HIV-1 infected cells positive for staining with the 2G12, 17b, and HIVIG Ab were not detected at any time point from day 3 through 7 among the CH077-infected cells but were seen at days 6 and 7 in NL4-3 infected CD4⁺ T cell cultures. These data suggested that HIV-1 envelope glycoproteins expressed on the surface of HIV-1 infected cells
25 could be recognized by mAb A32 earlier than by either 17b or 2G12 mAbs or by Env-specific Abs present in HIVIG. Moreover, these data suggest that expression of the HIV-1-derived surface epitopes on R5 transmitted/founder virus infected

CD4 T cells may differ from those expressed on X4 chronic virus infected CD4 T cells.

Monoclonal Antibody A32 can mediate ADCC. It was originally reported that A32 mAb does not mediate HIV-1 neutralizing activity (22, 34). Similarly, in our neutralization assays in both TZM-bl cells and in PBMC cultures, it has also been found that A32 mAb did not neutralize HIV-1 (data not shown). The next question asked was whether mAb A32 could mediate ADCC activity. The CEM.NKR_{CCR5} cell line was first utilized as a source of target cells upon their coating with recombinant Env proteins or as a chronically infected cell line. The recombinant Env proteins utilized to coat the CEM.NKR_{CCR5} cell represented the gp120 and gp140 sequence, respectively, of the transmitted/founder HIV *env* from subjects WITO4160 ("WITO") and CH040. A32 mAb was able to recognize both the gp120_{WITO} and to mediate ADCC activity expressed as % GzB activity (Fig. 3A). The maximum %GzB activity detectable with A32 was 52±8% at 0.156µg/ml of A32 mAb. At this concentration, its activity was almost 6 fold higher than that observed with mAb 2G12 (8±5.8%) and 17b (9±2%). Similar results were observed when the gp140_{CH040}-coated target cells were used (Fig. 3B). A32 was able to mediate a maximum of 40% GzB ADCC activity at the concentration of 0.156µg/ml that was 2.6-fold higher than that observed with mAb 17b at 40µg/ml (15% GzB). In contrast to WITO gp120 coated targets, no ADCC activity against the gp140_{CH040}-coated target cells was detected using mAb 2G12. It should be noted that in both settings the A32 ADCC activity displayed the previously described prozone phenomena with high concentrations of antibody inhibiting ADCC (21, 33).

A32 mAb is capable of mediating ADCC responses against HIV-1 infected target cells. Using the A1953_CEM.NKR_{CCR5} target cells the peak of the ADCC activity mediated by A32 mAb was 42%GzB at 0.625µg/ml (Fig. 3C). This was a 4-fold higher activity than what observed with 17b mAb at the concentration of

40µg/ml. Moreover, ADCC activity mediated by A32 mAb was detected at concentrations of 50 and 5µg/ml when NL-LucR.T2A-BaL.ecto infected PB CD4⁺ T cells obtained from a healthy HIV-1 seronegative were used to generate target cells as described in the method section. (Fig. 4). The NK to target cell ratio of 40:1 was required to detect an activity comparable to what observed when the HIVIG preparation was tested at 50µg/ml. These levels of granzyme B release compared to those obtained using recombinant envelope protein-coated and chronically infected target cells indicate that higher concentrations of A32 and effector cells are required to eliminate HIV-1-infected CD4⁺ T cells.

Inhibition of ADCC-mediating Ab activity in plasma from infected individuals by the Fab of A32 antibody. To determine whether the epitope recognized by A32 mAb was also targeted by ADCC responses in chronically infected individuals, a strategy was devised where target cells were preincubated with an A32 Fab fragment to prevent the subsequent binding and ADCC activity of Ab present in the plasma of HIV-1-infected subjects (Fig. 5). The pre-incubation of the target cells with 1 and 0.1µg/ml of the A32 Fab blocked 92 and 80% of the A32 mAb ADCC activity when 10µg/ml of the A32 were used, respectively (Fig. 5A). Based on this experiment, the ability of the A32 Fab to inhibit ADCC activity of Ab present in the plasma of HIV-1 infected individuals was determined. A test was made of plasma samples from donors recruited in the cohort of chronically infected individuals representing a range of ADCC-mediating Ab responses (Fig. 5B). A 10µg/ml concentration of A32 Fab was used to pre-incubate target cells in order block the A32 binding on the A1953_CEM.NKR_{CCR5} cells. The plasma samples from HIV-1 infected individuals were added to the target cells after this initial incubation at two dilutions representing the peak and subsequent ADCC reactivity for each sample. Observed was a range of inhibitory activity for the peak of the response due to the A32 Fab from 19% to 90% in the lower dilution (average ± standard deviation =

56±24). In each individual, a higher level of inhibition of the ADCC activity was at the higher dilution ranging from 48 to 96% (75±16); the majority of ADCC activity (>50%GzB activity) was also inhibited in 12 out of 14 patients (86%) at the lowest dilution. In both instances, the ADCC activity in the plasma samples was significantly lower when the target cells were pre-incubated with the A32-Fab (Wilcoxon matched-pairs signed rank test $p = <0.0001$), whereas pre-incubation with control Ab did not inhibit the observed level of ADCC activity (Wilcoxon matched-pairs signed rank test $p = <0.4556$). (See also Figs. 6-8.)

10 In summary, in this study, it was demonstrated that the HIV-1 Env gp120 mAb A32 binds to the surface of transmitted/founder HIV-1-infected CD4⁺ T cells earlier in the course of *in vitro* infection than the gp120 Env mAbs 17b and 2G12. Moreover, A32 mAb was able to mediate ADCC activity that was 4-6-fold higher than the other two anti-gp120 mAbs when either gp120-coated or HIV-1-15 infected target CD4⁺ cells were used. Finally, it was demonstrated that antibodies that are blocked by A32 Fab comprise a majority of ADCC-mediating Ab responses elicited during the course of HIV-1 infection.

It has been previously reported that mAb A32 can be expressed on the surface of HIV-1 infected cells during the latter stages of HIV-1 mediated CD4⁺ T cell line fusion (10). MAbs directed toward the C-terminus, V3 loop and CD4 binding domain of the gp120 display a broad spectrum of binding capacity to the HIV-1 envelope glycoproteins expressed on the surface of infected cells (36). 17b and 2G12 mAbs were previously shown to stain the surface of a HeLa cell line that produced replication-defective HXB2 virions and inhibit cell-to-cell fusion 20 (10). In this current study, activated peripheral blood CD4⁺ T lymphocytes were used in the experiments to more closely replicate what may occur *in vivo*. It was observed that A32 mAb was capable of binding to CD4⁺ T cells infected with both primary (transmitted/founder) and laboratory adapted HIV-1 isolates before

potent neutralizing 17b and 2G12 mAb could bind. This was somewhat surprising and suggested that *in vitro* cell surface expression of gp120 Env epitopes may be differentially regulated. It was important to determine that A32 provided the earliest positive signal to identify envelope expression on the surface of HIV-1 infected cells. A32 mAb binds to a conformation gp120 determinant that involves the C1 region (34). A32 is non-neutralizing and the reason for this has been attributed to the inability of A32 to bind to virions, i.e. the antibody has been suggested to not bind to the native trimer (34). The work on PB CD4+ T cells and the previous work of Finnegan et al. clearly demonstrate that mAb A32 does bind to the surface of the native trimer on the surface of HIV-1-infected cells. Moreover, these data suggest that the conformation of the Env trimer with regard to the conformational C1 region is dramatically different on virions compared to virus-infected cells.

The ability of mAb A32 to mediate ADCC was further explored using a variety of target cell populations including gp120-coated CD4+ T cell line cells, chronically infected CD4+ T cells using the A1953 isolate and PB CD4+ T cells infected with transmitted/founder HIV-1 infectious molecular clones. The ADCC-mediating activity observed for 2G12 using the gp120-coated target cells was comparable to that previously reported (32) and the paucity of ADCC reactivity observed for 17b confirms previous observations indicating the need for sCD4 triggering of some Envs for exposure of the 17b CCR5-binding site epitope (30). The ability of A32 to mediate ADCC activity has also been confirmed in recent studies exploring the role of CD4i mAbs in ADCC responses. The ability of A32 to mediate ADCC activity has also been confirmed by recent studies performed by George Lewis and Anthony DeVico while exploring the role of CD4i mAbs in ADCC responses. Importantly, observed was a 4-6 fold higher ADCC activity of A32 mAb to mediate ADCC activity compared to 17b and 2G12 mAbs and was comparable to ADCC mediated by HIV+ immunoglobulin (HIVIG®).

Most importantly, the presence of A32-like Ab responses was observed in the serum of subjects with chronic HIV-1 infection. It was observed that A32-like Ab mediated the majority of the total ADCC activity in 86% of the chronically infected subjects when the highest dilution was tested.

5 It will be appreciated from a reading of this disclosure that if mAb A32 is shown to mediate a component of prevention of acquisition in passive protection trials in non-human primates, then it will be important to design immunogens that could enhance the antigenicity of the gp120 region for mAb A32 binding in order to improve HIV-1 vaccine candidates.

10

EXAMPLE 2

Specificity of RV144 induced antibodies is shown in Fig. 9 by measuring the blocking of RV144 vaccinee purified plasma IgG binding to Immunogen (A244) Env gp120 protein by gp120 mAbs in a SPR assay. The binding of RV144 15 IgG to A244 gp120 was blocked by A32 C1 mAb (66%), the neutralizing V2 conformational mAb 697-30D (19%), the broadly neutralizing quaternary mAb CH01 (24%), and the Tier 1 restricted V3 neutralizing antibody 19b (42%). n= number of vaccinee IgG studied.

20 RV144 induced antibody responses on C1 (A32), V2 (697-30D), V3 (19b) and at or near the conformational-Quaternary V2-V3 epitope (CH01) are shown in Fig. 10.

Quaternary Abs require a V2 N-linked glycosylation site at V2 loop aa 160 (N160) for binding and for neutralization. Therefore, a search was made in 25 serum for Abs that bound better to N160 than to N160K A244 gp120.

Quaternary mAb CH01 binding to A244 gp120 is abrogated by N160K mutation, as shown in Fig. 11.

Differential binding of RV144 vaccinee purified IgG to A244 WT versus A244g N160K gp120 (see Fig. 12). SPR binding responses of RV144 plasma IgG to A244-N160 WT versus A244-N160K mutant were measured on a BIAcore 4000. IgG samples that gave higher sensitivity (>25% reduction) to N160K mutation are circled in red. About 20% of the RV144 vaccinee IgG showed N160K sensitivity, which is compatible to specificities of neutralizing conformational-quaternary gp120 antibodies.

Summary of Results

- The binding specificities of RV144 IgGs map to epitopes within C1, V2, and V3 domains of gp120
- C1 (A32 blocking), V3 (19b blocking) and V2 (697D) specificities were predominant
- About 20% of RV144 IgG samples showed sensitivity to N160K mutation
- Blocking with CH01 mAb and sensitivity to V2-N160K mutation suggested that V2-V3 quaternary antibodies may have been induced in select RV144 vaccinees

* * *

All documents and other information sources cited above are hereby incorporated in their entirety by reference.

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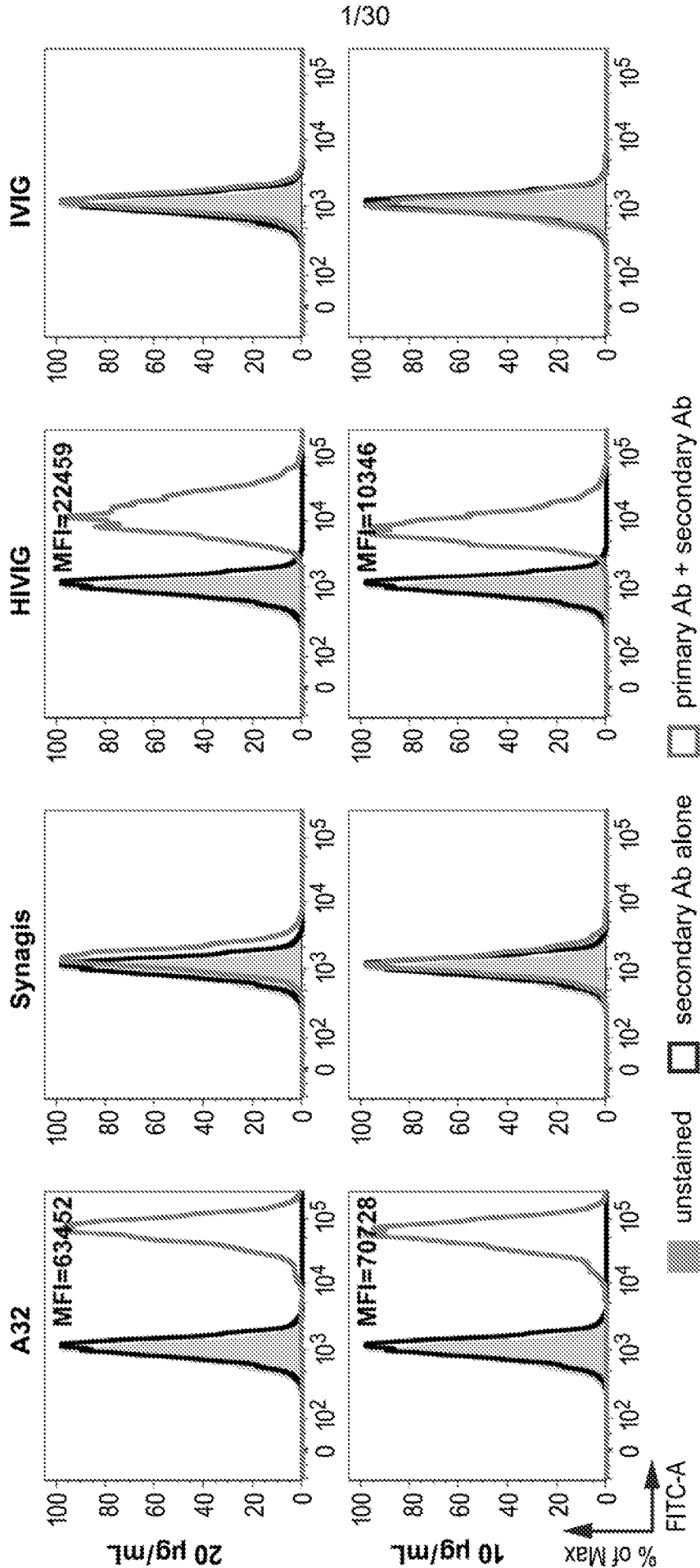
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WHAT IS CLAIMED IS:

1. An isolated human immunodeficiency virus type 1 (HIV-1) envelope immunogen that induces the production in a subject of antibodies the
5 binding of which to said immunogen is blocked by monoclonal antibody A32 or binding fragment thereof.
2. The immunogen according to claim 1 wherein said binding
fragment is a Fab fragment.
10
3. A composition comprising the immunogen according to claim 1
and a carrier.
4. An isolated nucleic acid encoding said immunogen according to
15 claim 1.
5. The nucleic acid according to claim 4, wherein said nucleic acid is
present in a vector.
- 20 6. A composition comprising the nucleic acid according to claim 1
and a carrier.
7. A method of inducing an immune response against HIV-1
comprising administering a subject in need thereof an amount of said immunogen
25 according to claim 1 sufficient to effect said induction.
8. The method according to claim 7 wherein said subject is a human.

9. The method according to claim 7 wherein a nucleic acid encoding said immunogen is administered to said subject under conditions such that said nucleic acid is expressed and said immunogen is thereby produced.

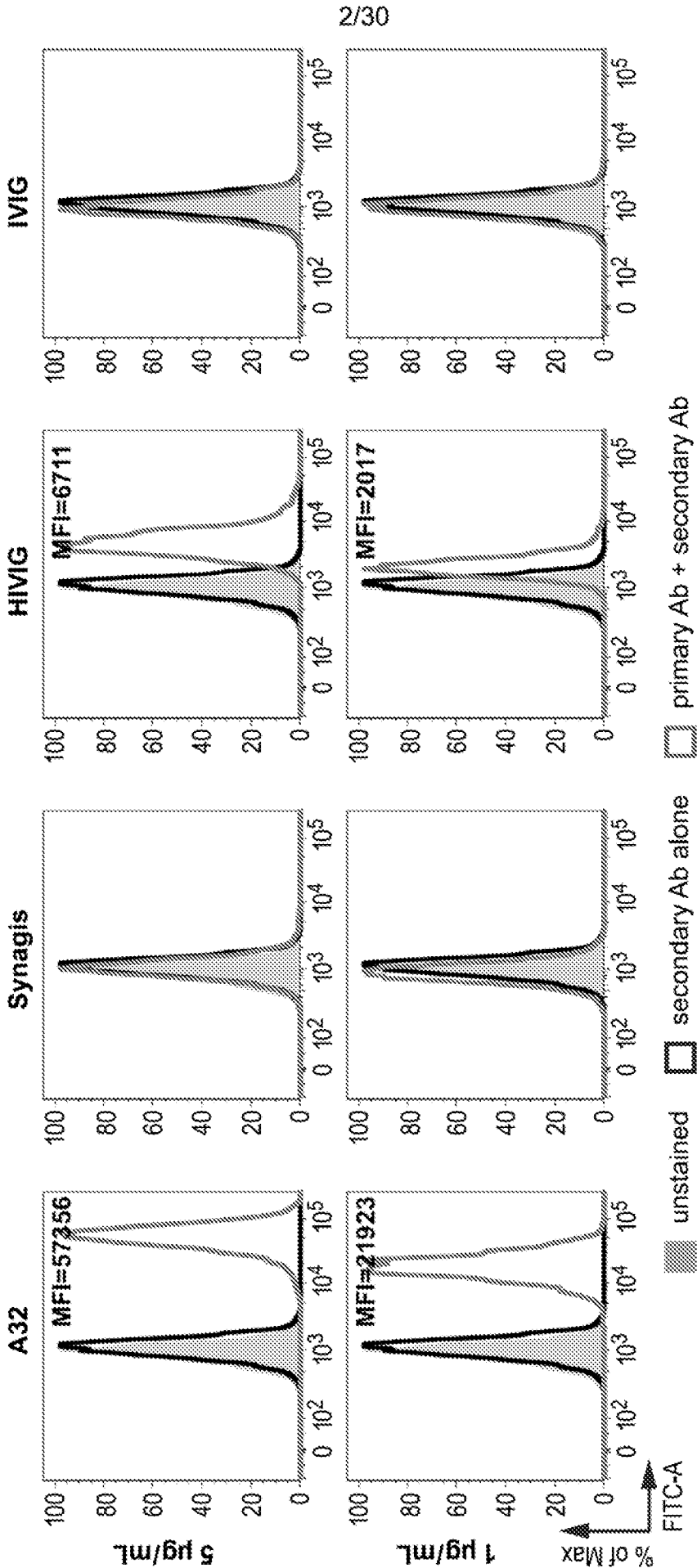
Surface Expression for A1953_SupT1 Cells



Detection of Env expression on the cellular membrane of the A1953_SupT1 cell line.

Figure 1

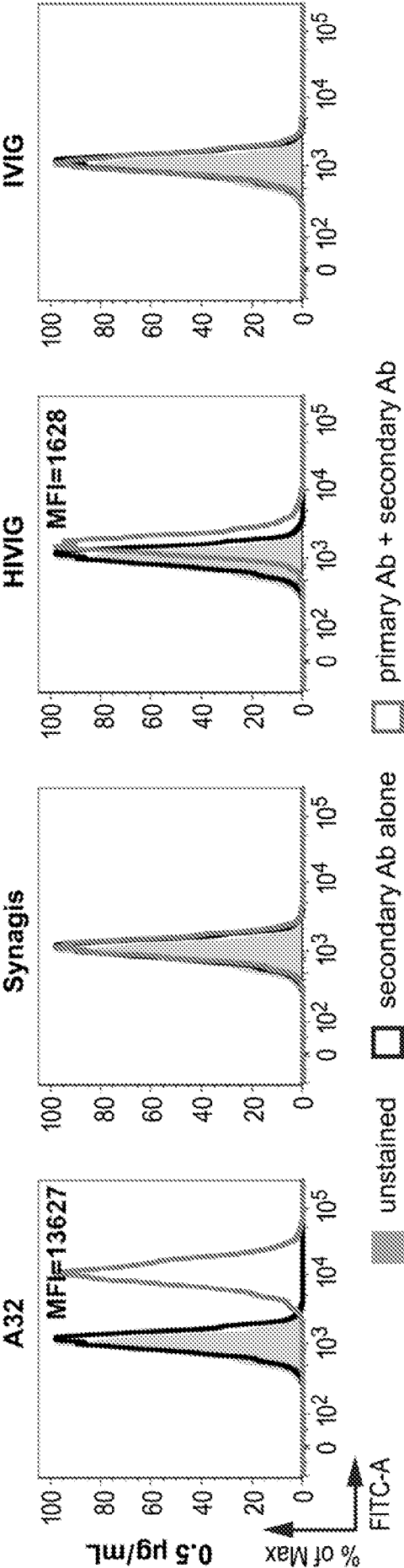
Surface Expression for A1953_SupT1 Cells



Detection of Env expression on the cellular membrane of the A1953_SupT1 cell line.

Figure 1 cont'd

Surface Expression for A1953_SupT1 Cells

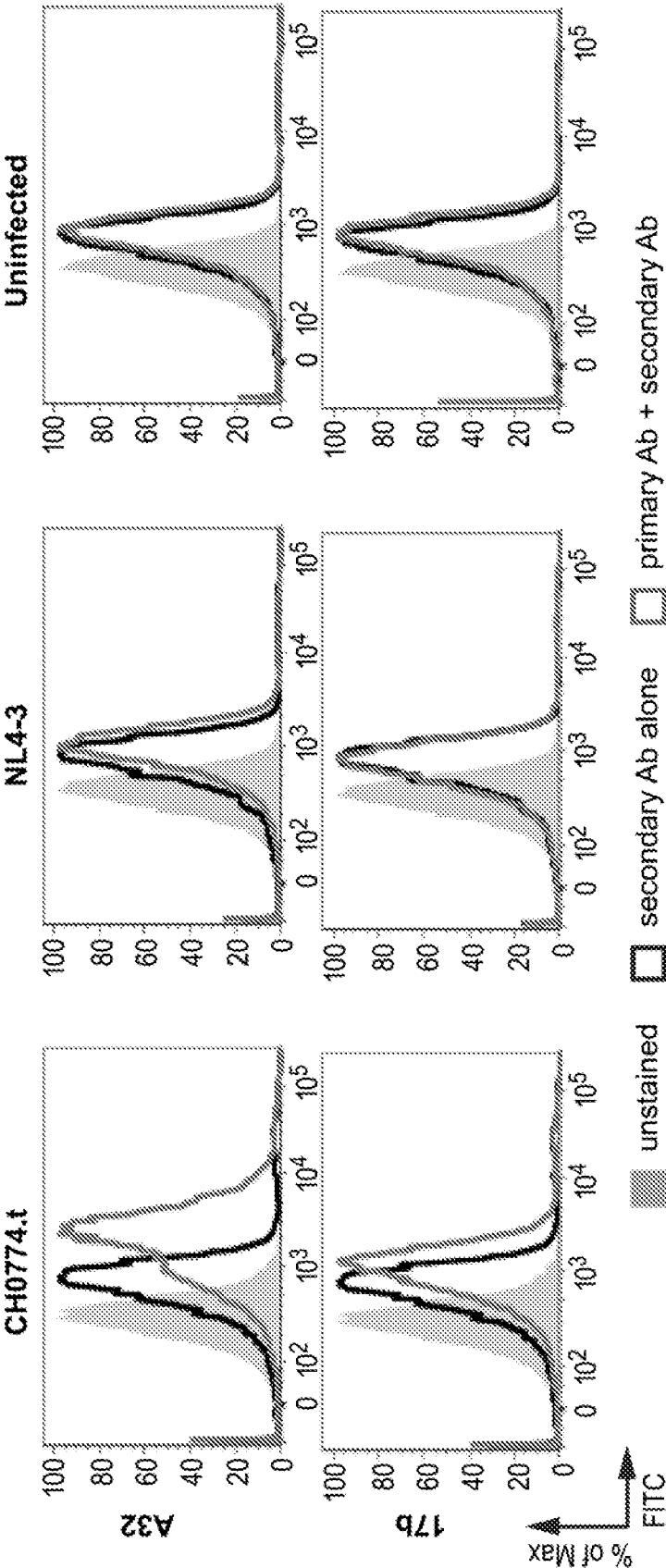


Detection of Env expression on the cellular membrane of the A1953_SupT1 cell line.

Figure 1 cont'd-2

Surface Expression for HIV-infected CD4⁺ T cells

Day 3



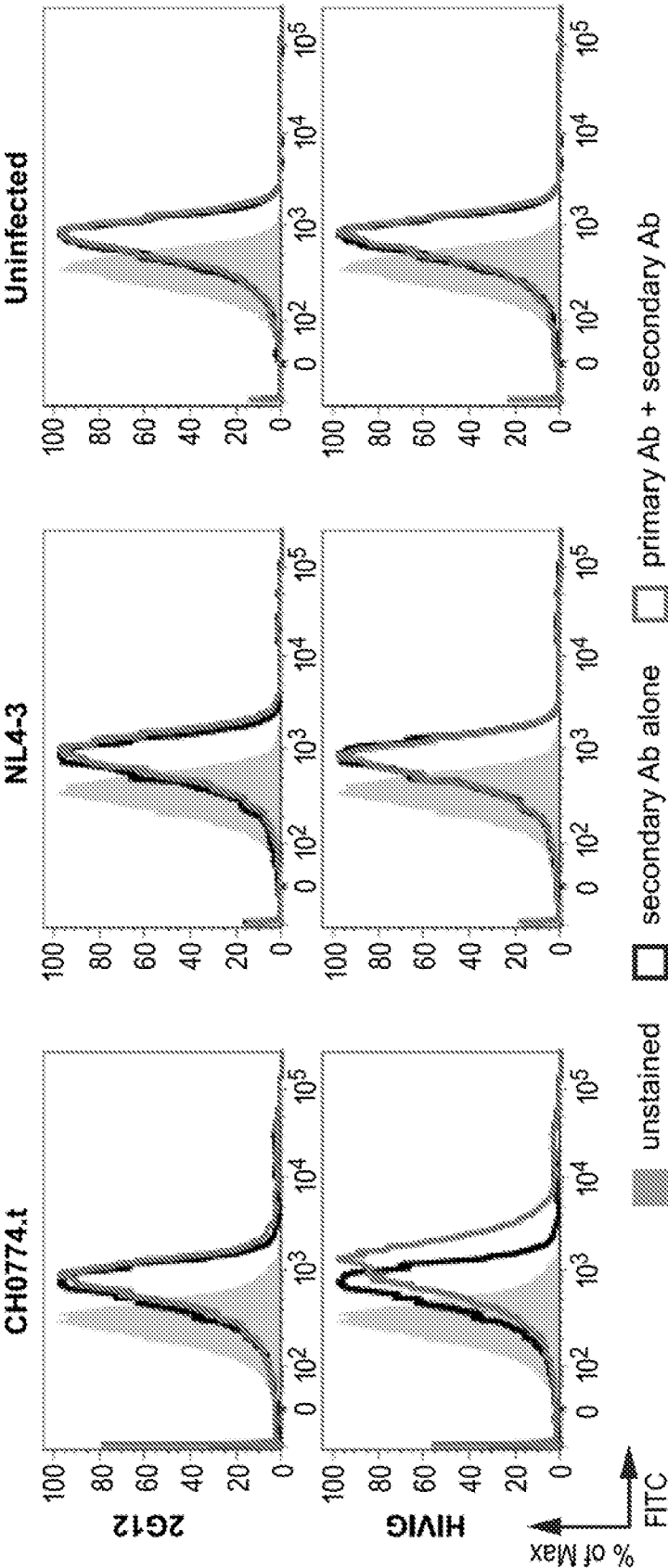
Detection of Env expression on the cellular membrane of HIV-infected CD4⁺ T cells.

Figure 2

5/30

Surface Expression for HIV-infected CD4⁺ T cells

Day 3

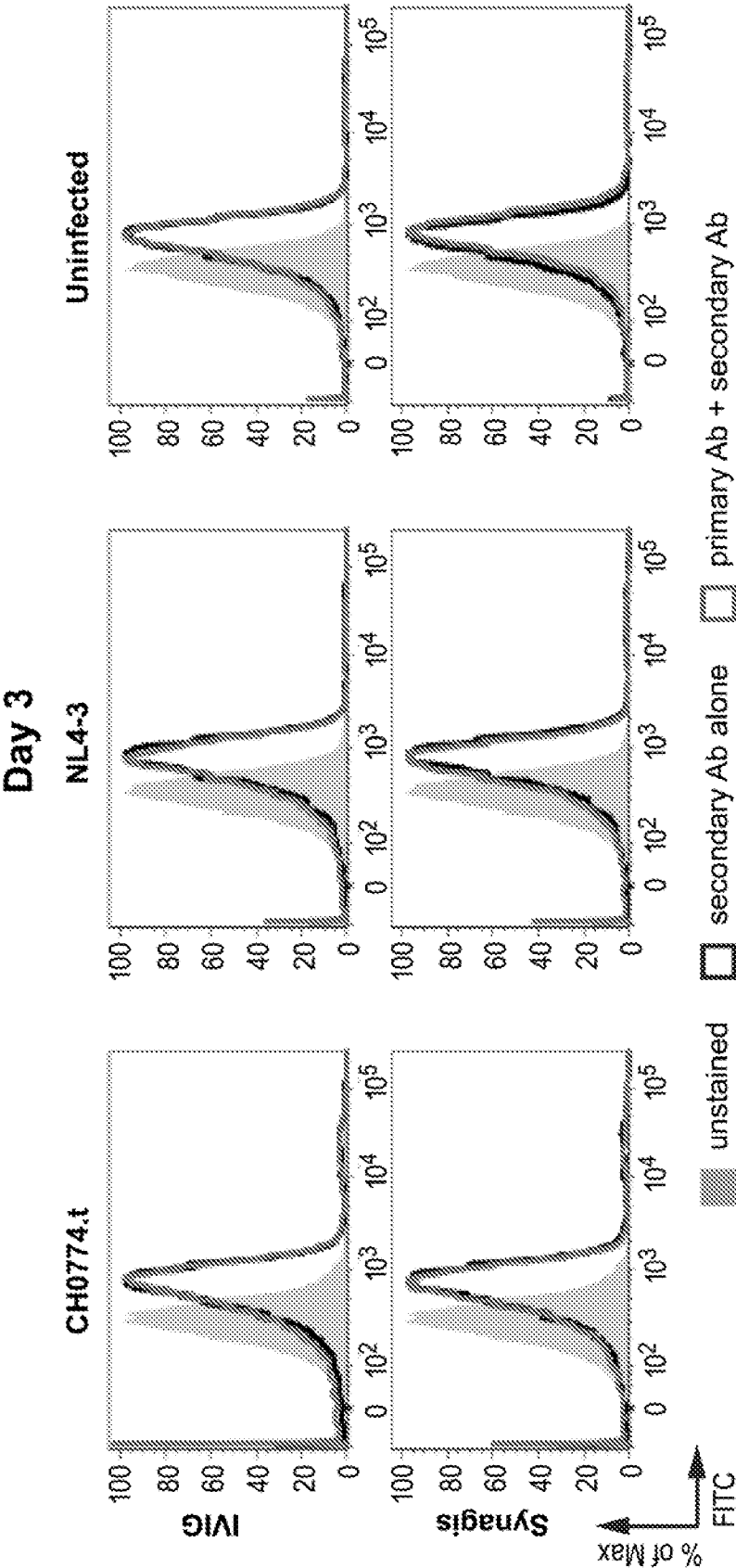


Detection of Env expression on the cellular membrane of HIV-infected CD4⁺ T cells.

Figure 2 cont'd

6/30

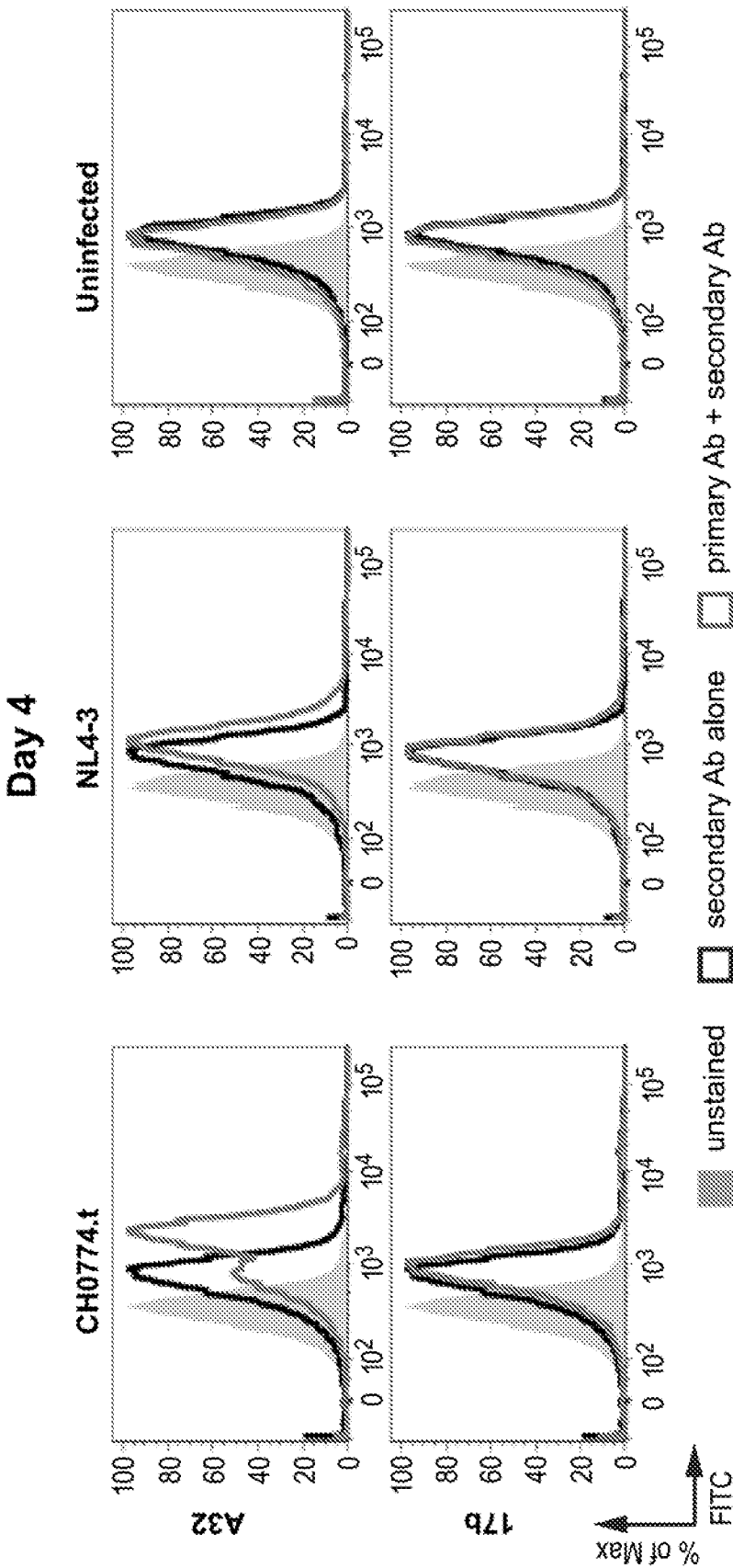
Surface Expression for HIV-infected CD4⁺ T cells



Detection of Env expression on the cellular membrane of HIV-infected CD4⁺ T cells.

Figure 2 cont'd-2

Surface Expression for HIV-infected CD4⁺ T cells

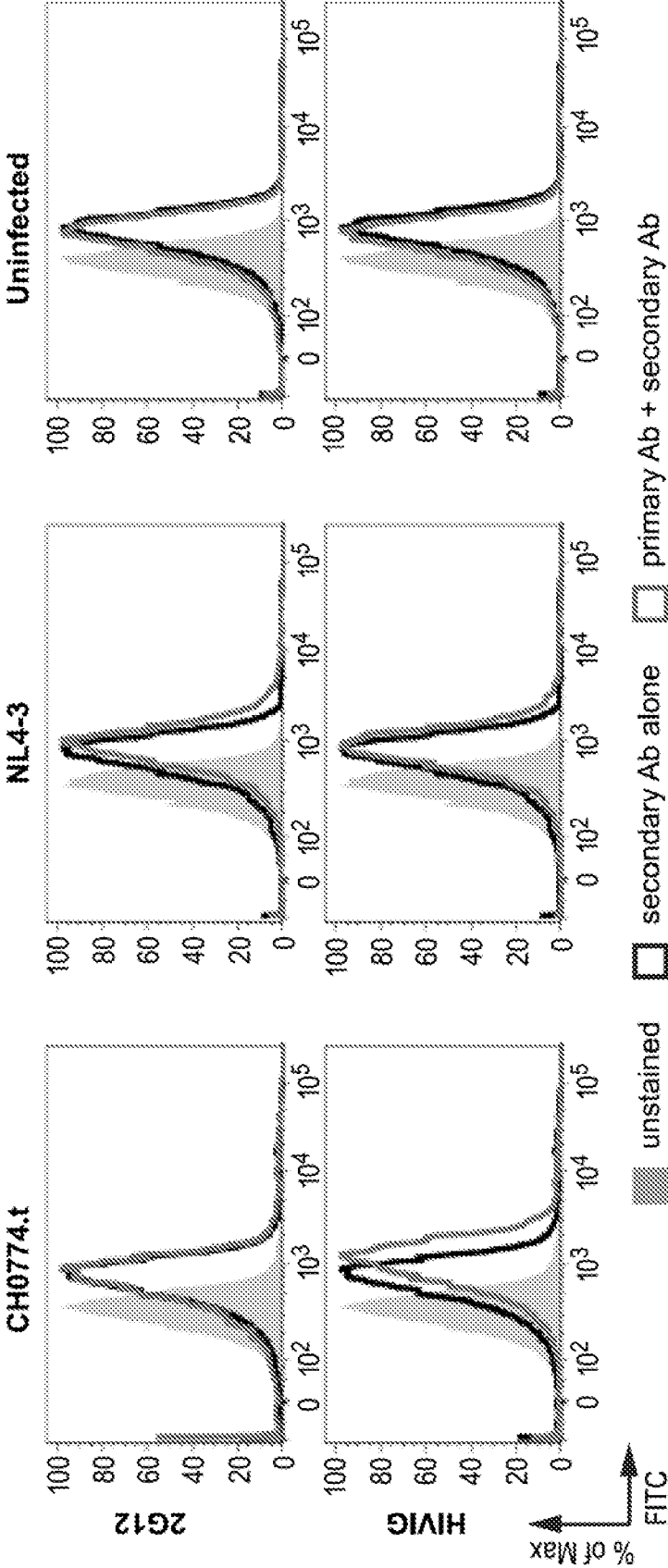


Detection of Env expression on the cellular membrane of HIV-infected CD4⁺ T cells.

Figure 2 cont'd-3

Surface Expression for HIV-infected CD4⁺ T cells

Day 4

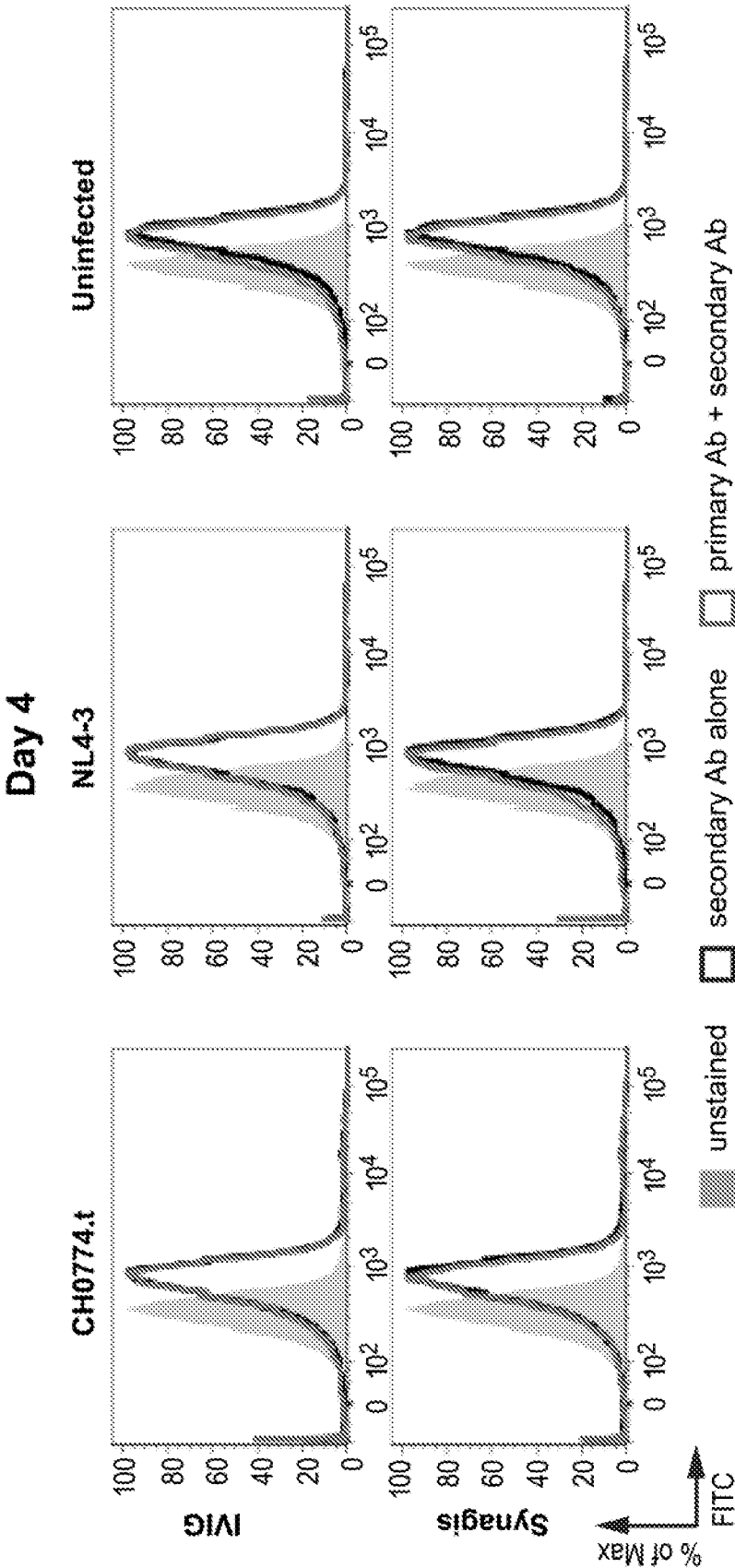


Detection of Env expression on the cellular membrane of HIV-infected CD4⁺ T cells.

Figure 2 cont'd-4

9/30

Surface Expression for HIV-infected CD4⁺ T cells



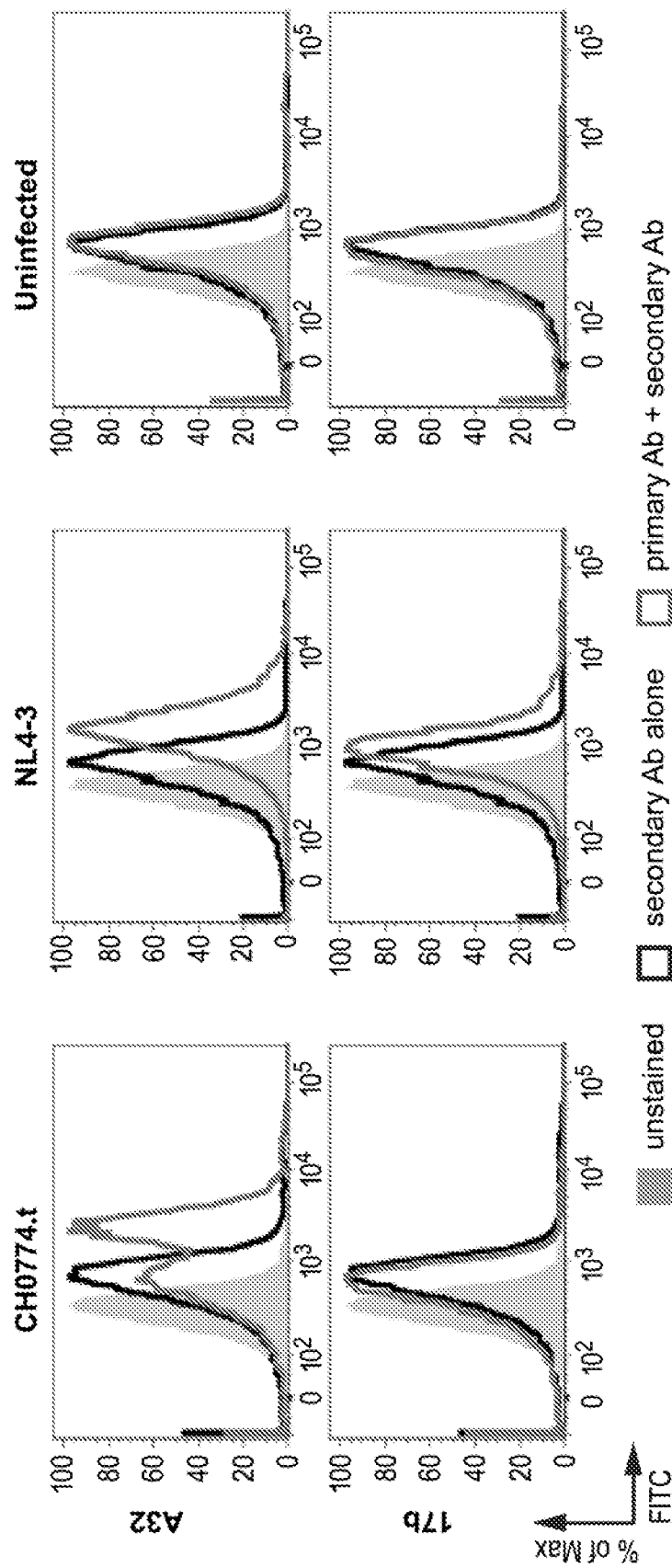
Detection of Env expression on the cellular membrane of HIV-infected CD4⁺ T cells.

Figure 2 cont'd-5

Surface Expression for HIV-infected CD4⁺ T cells

Day 5

10/30



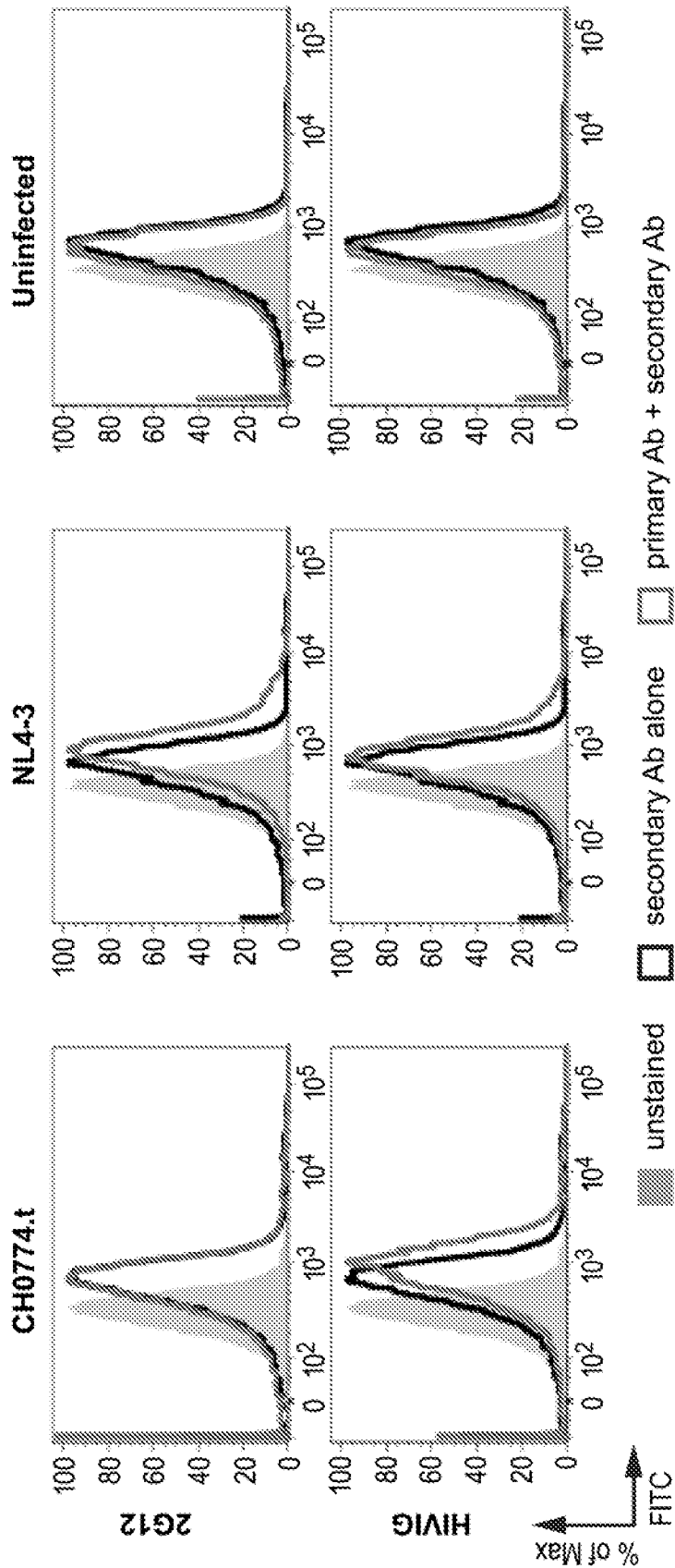
Detection of Env expression on the cellular membrane of HIV-infected CD4⁺ T cells.

Figure 2 cont'd-6

11/30

Surface Expression for HIV-infected CD4⁺ T cells

Day 5



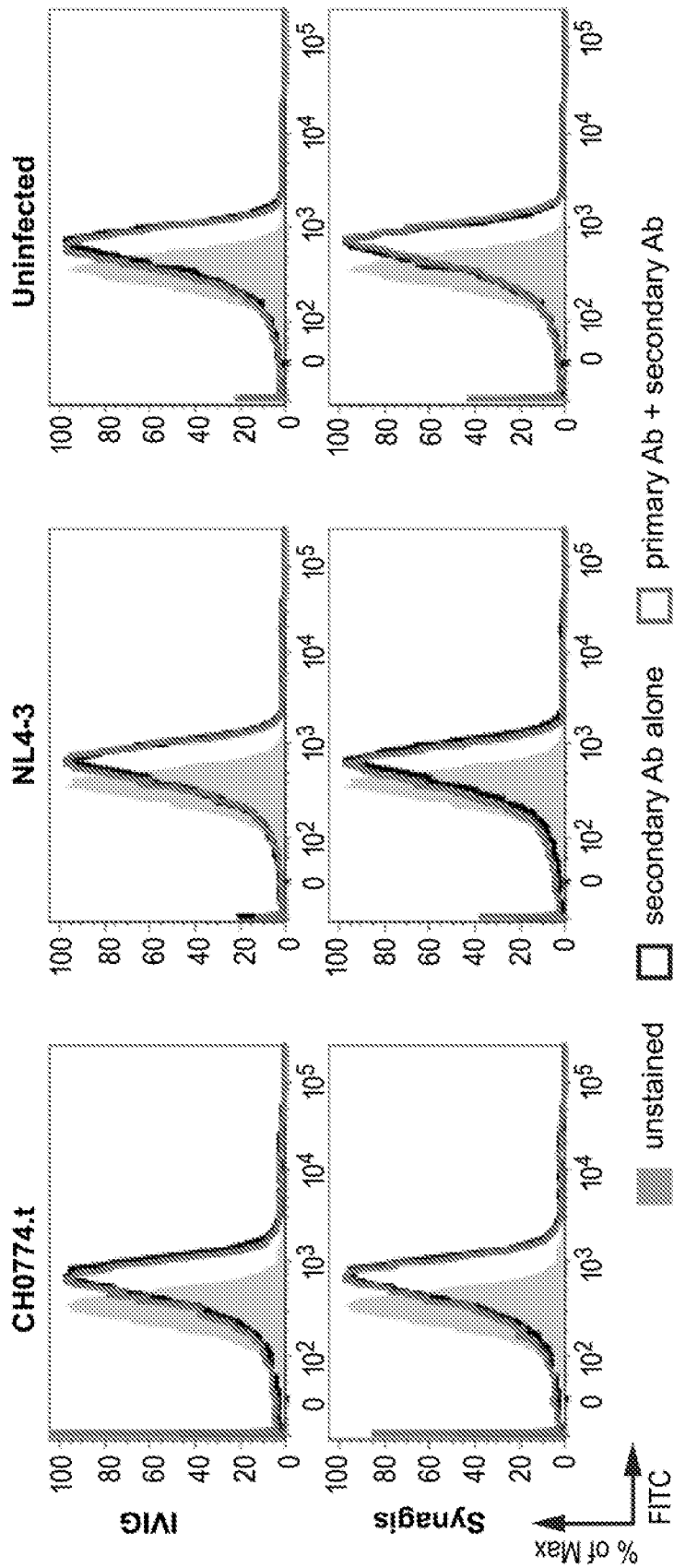
Detection of Env expression on the cellular membrane of HIV-infected CD4⁺ T cells.

Figure 2 cont'd-7

12/30

Surface Expression for HIV-infected CD4⁺ T cells

Day 5

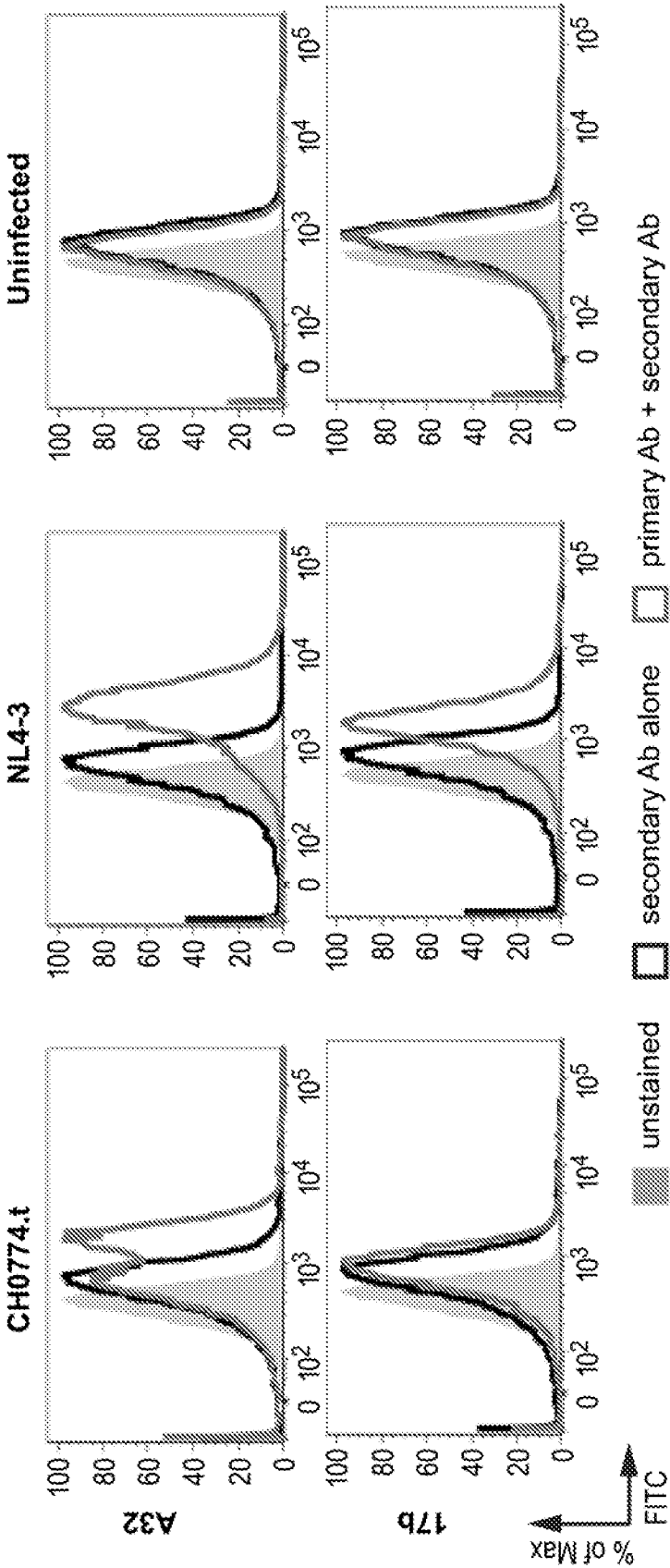


Detection of Env expression on the cellular membrane of HIV-infected CD4⁺ T cells.

Figure 2 cont'd-8

Surface Expression for HIV-infected CD4⁺ T cells

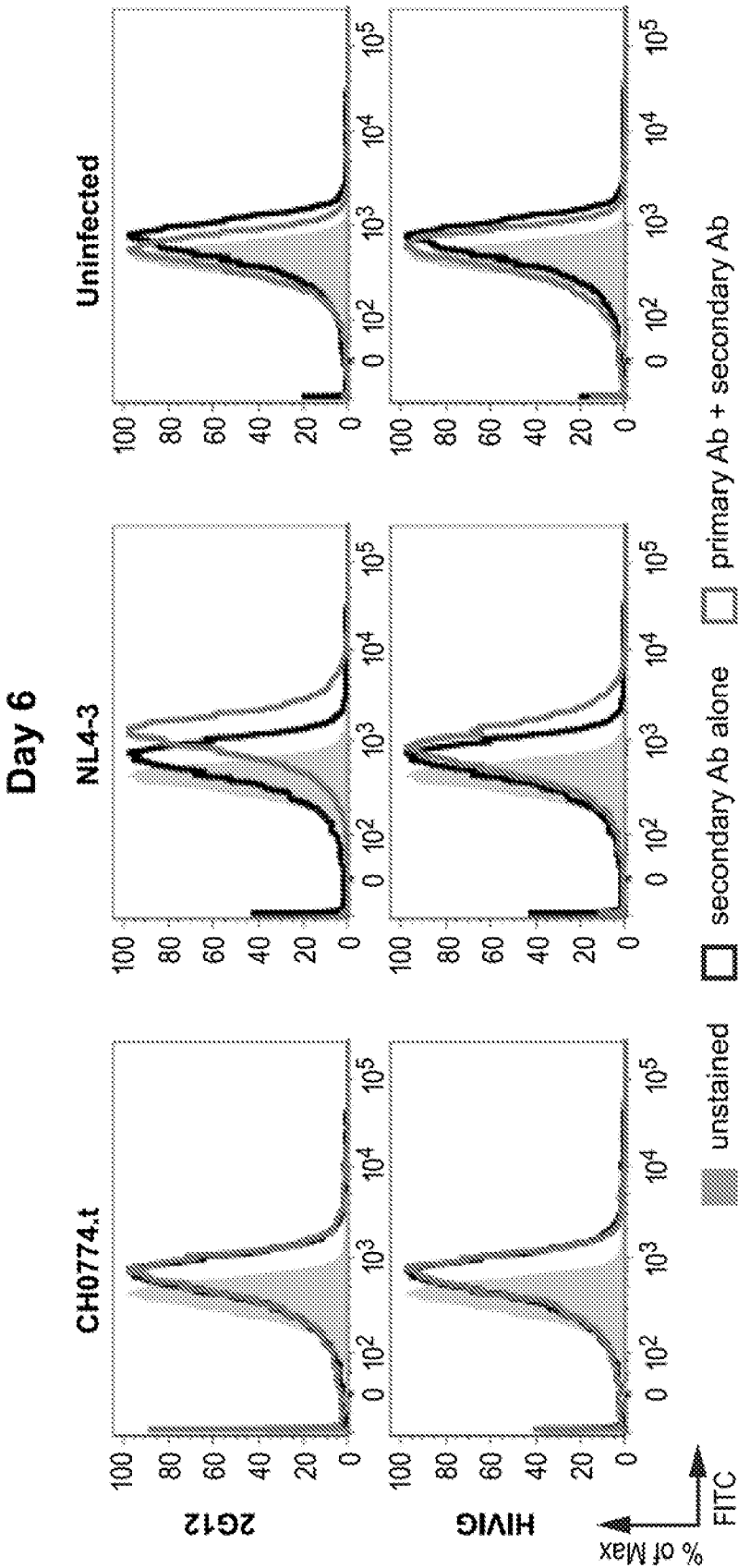
Day 6



Detection of Env expression on the cellular membrane of HIV-infected CD4⁺ T cells.

Figure 2 cont'd-9

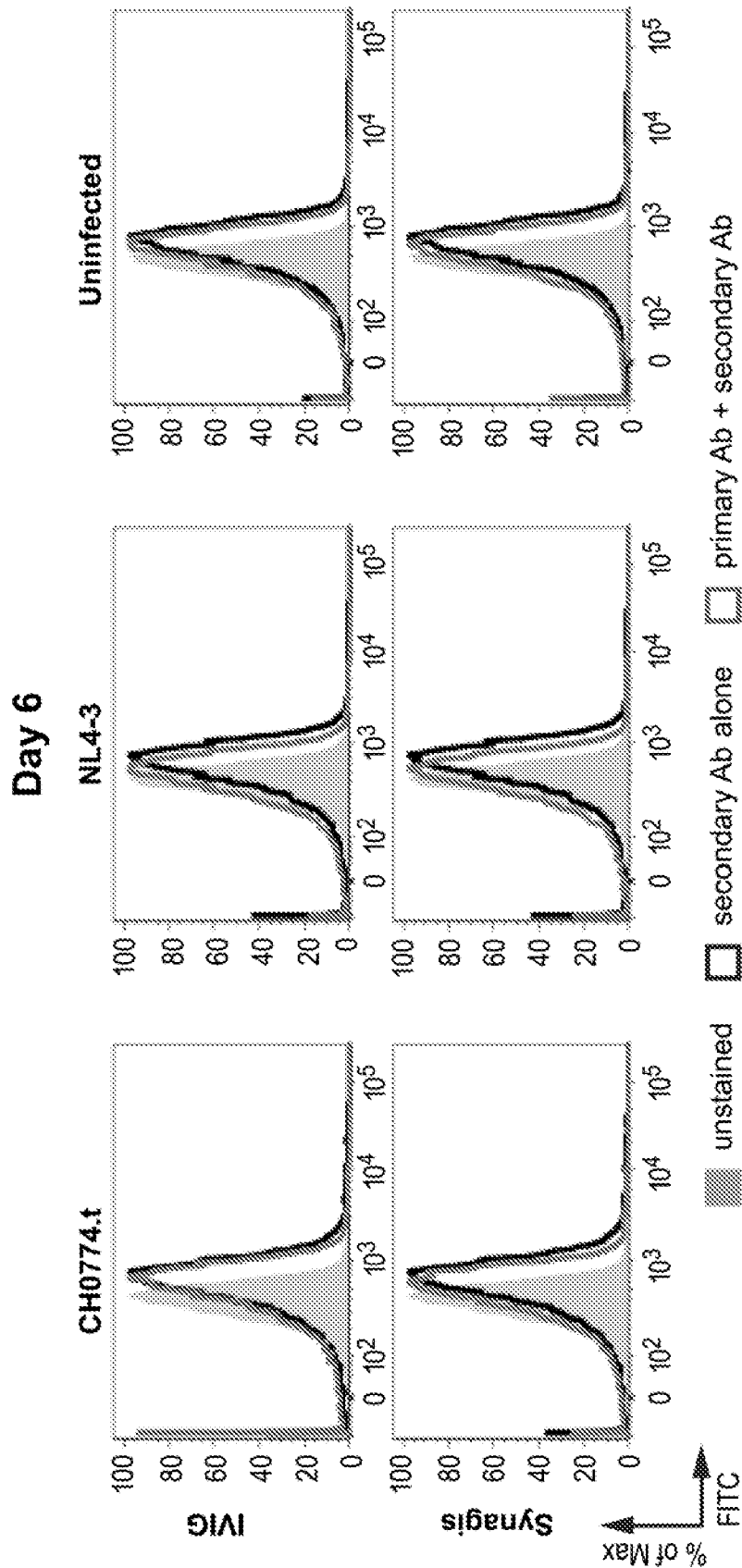
Surface Expression for HIV-infected CD4⁺ T cells



Detection of Env expression on the cellular membrane of HIV-infected CD4⁺ T cells.

Figure 2 cont'd-10

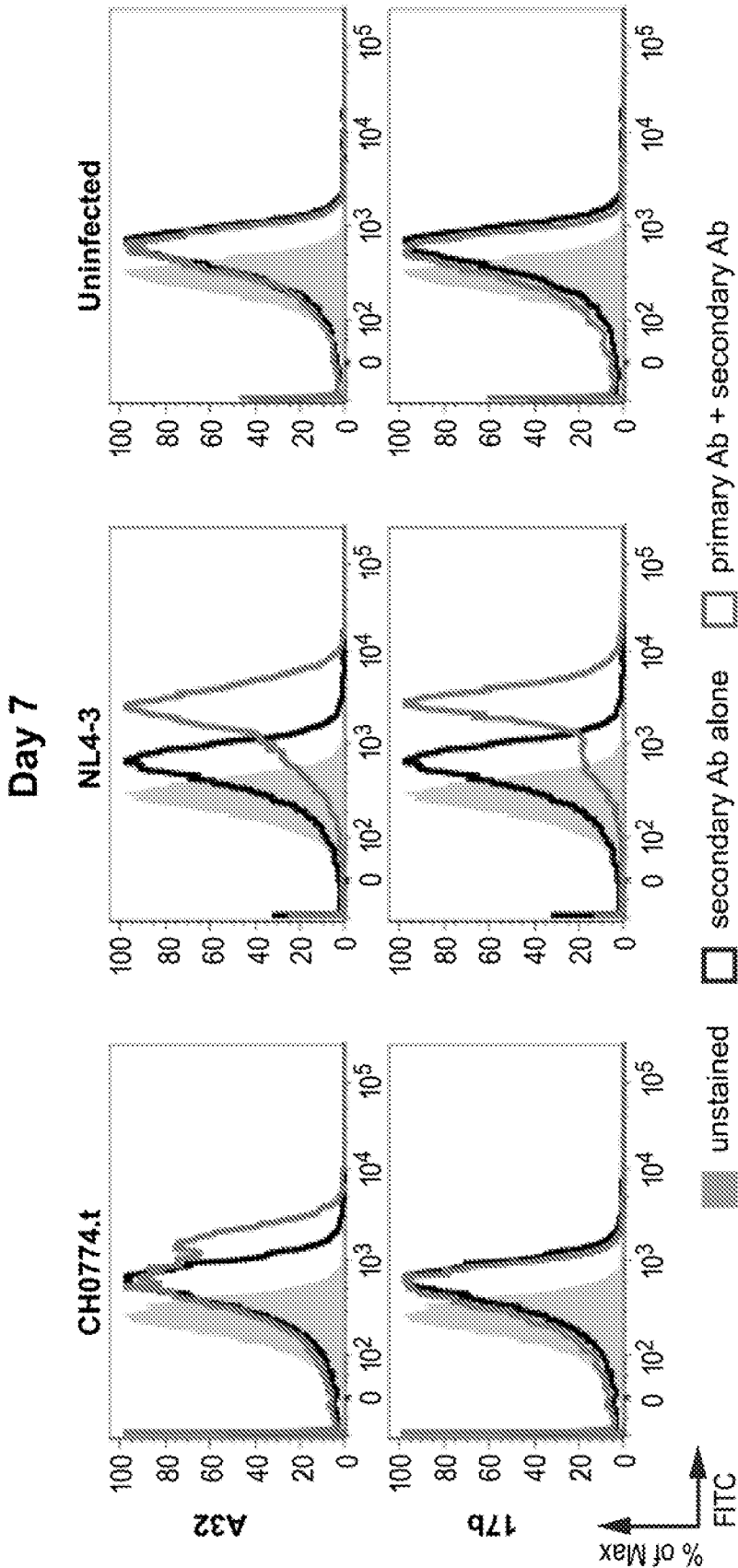
Surface Expression for HIV-infected CD4⁺ T cells



Detection of Env expression on the cellular membrane of HIV-infected CD4⁺ T cells.

Figure 2 cont'd-11

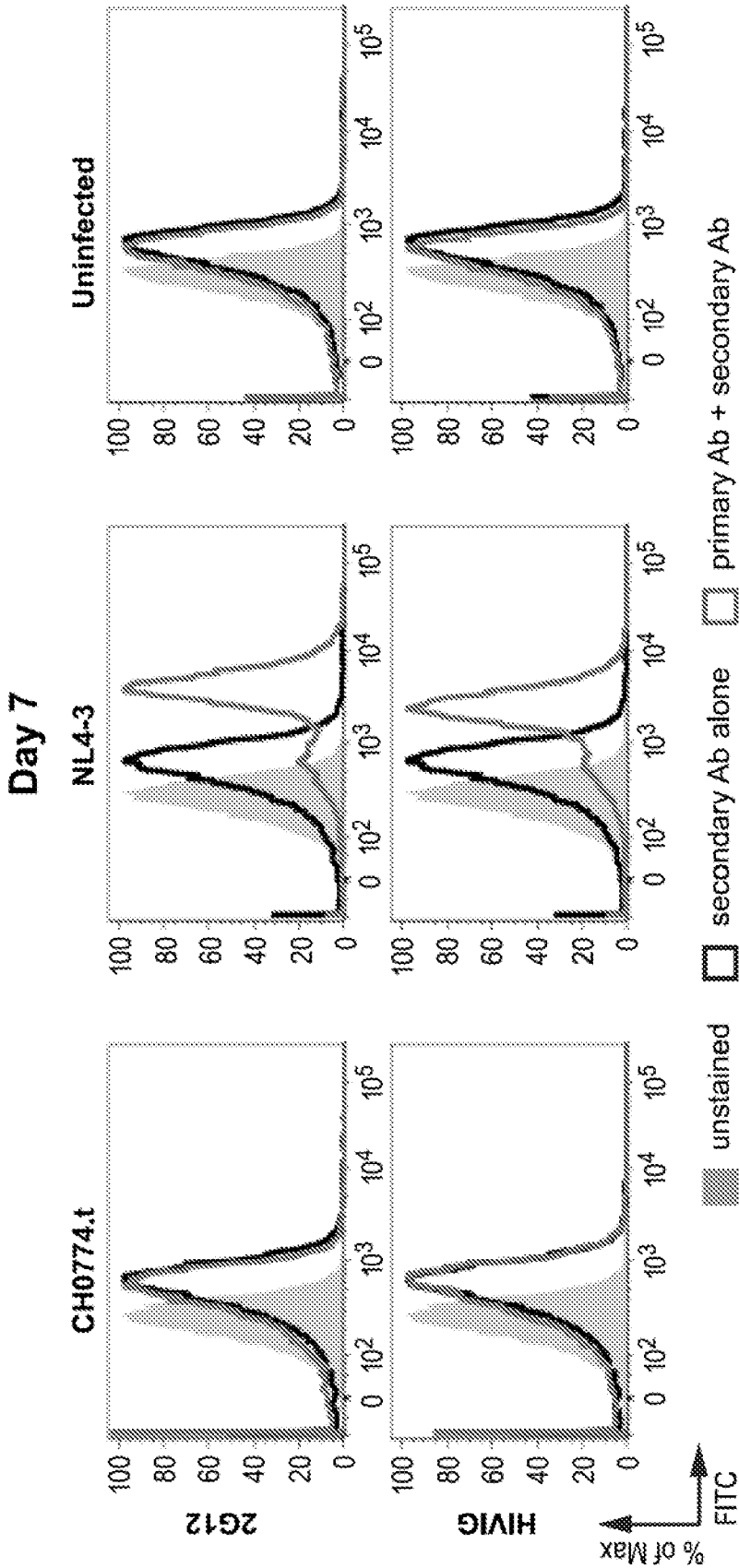
Surface Expression for HIV-infected CD4⁺ T cells



Detection of Env expression on the cellular membrane of HIV-infected CD4⁺ T cells.

Figure 2 cont'd-12

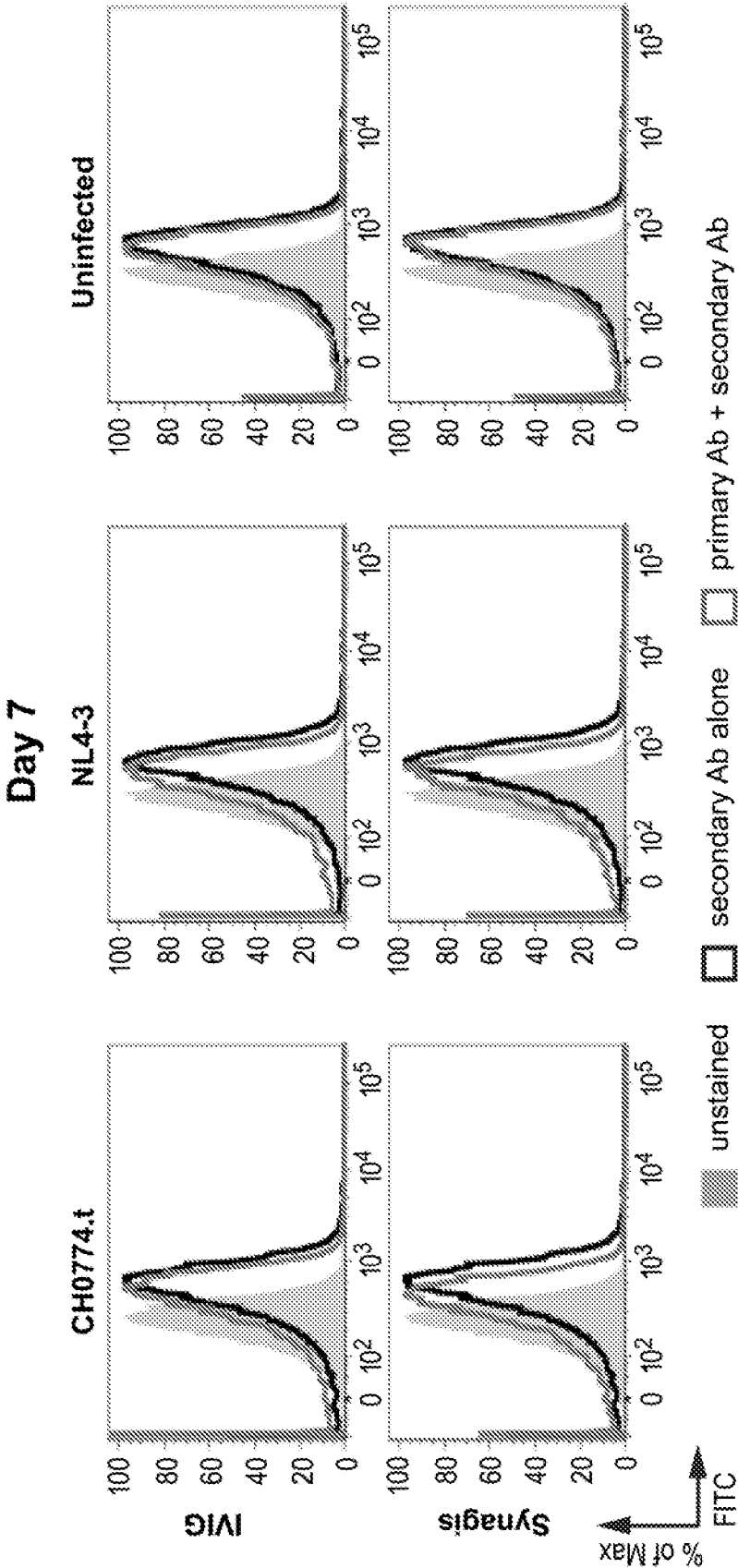
Surface Expression for HIV-infected CD4⁺ T cells



Detection of Env expression on the cellular membrane of HIV-infected CD4⁺ T cells.

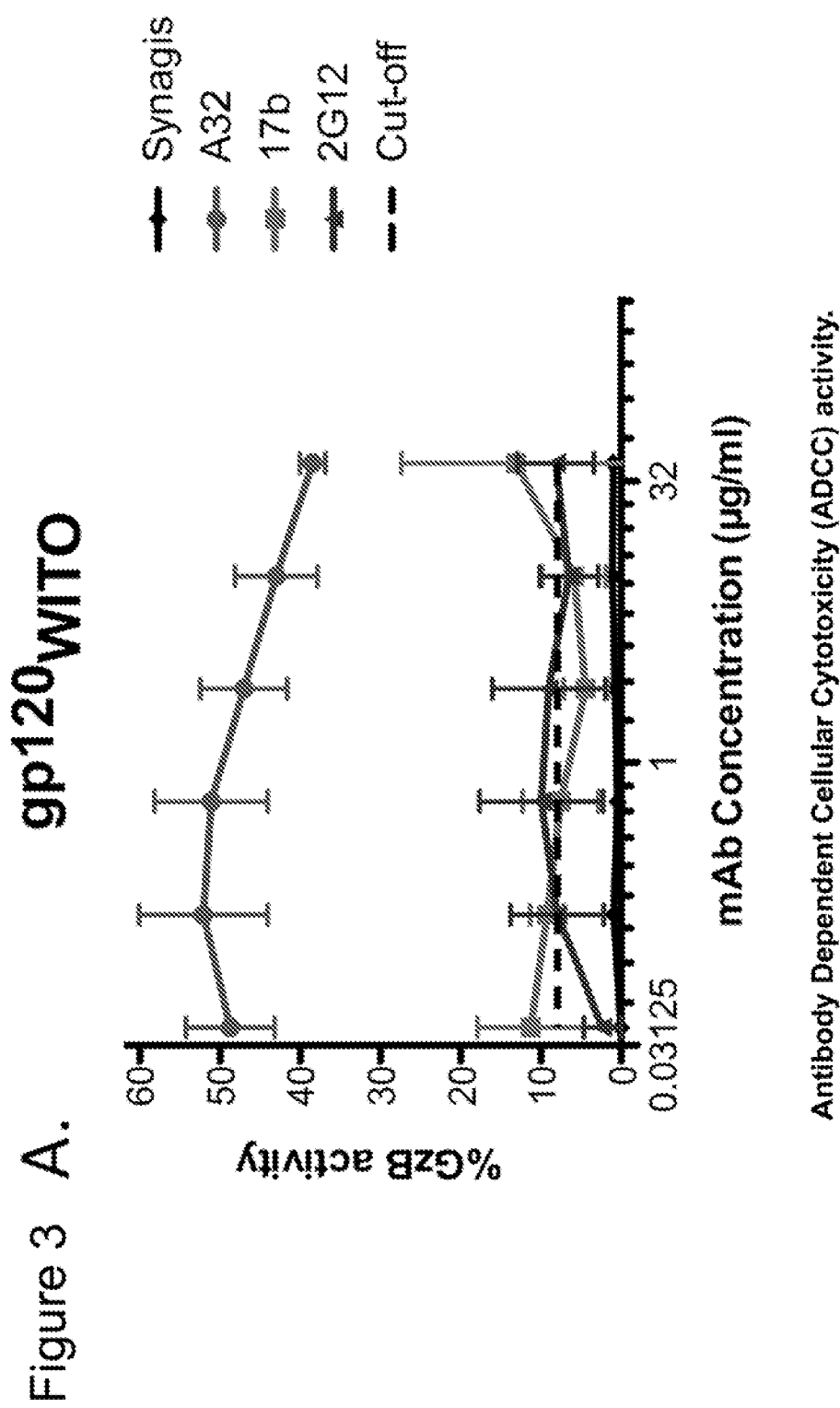
Figure 2 cont'd-13

Surface Expression for HIV-infected CD4⁺ T cells



Detection of Env expression on the cellular membrane of HIV-infected CD4⁺ T cells.

Figure 2 cont'd-14



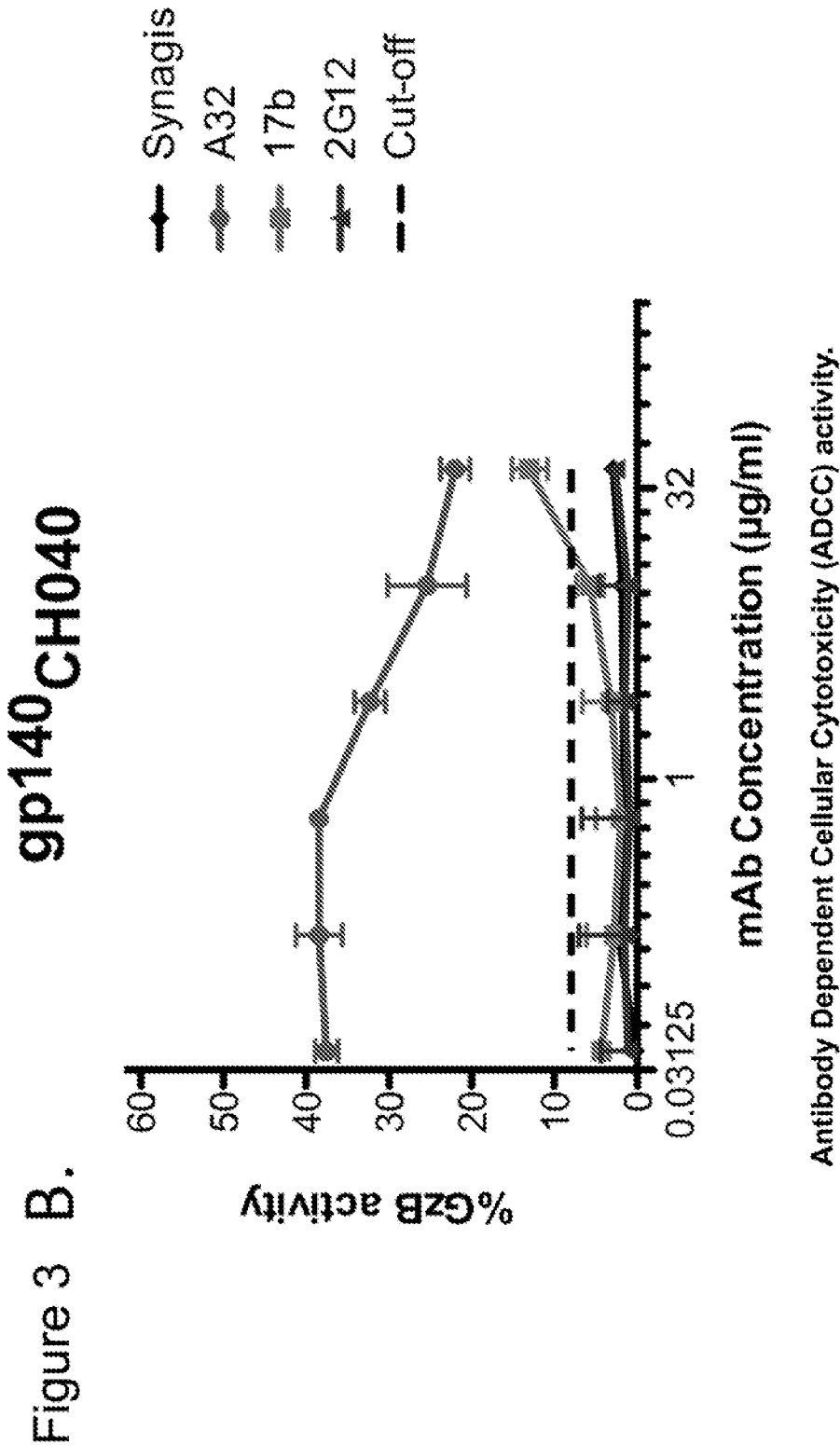
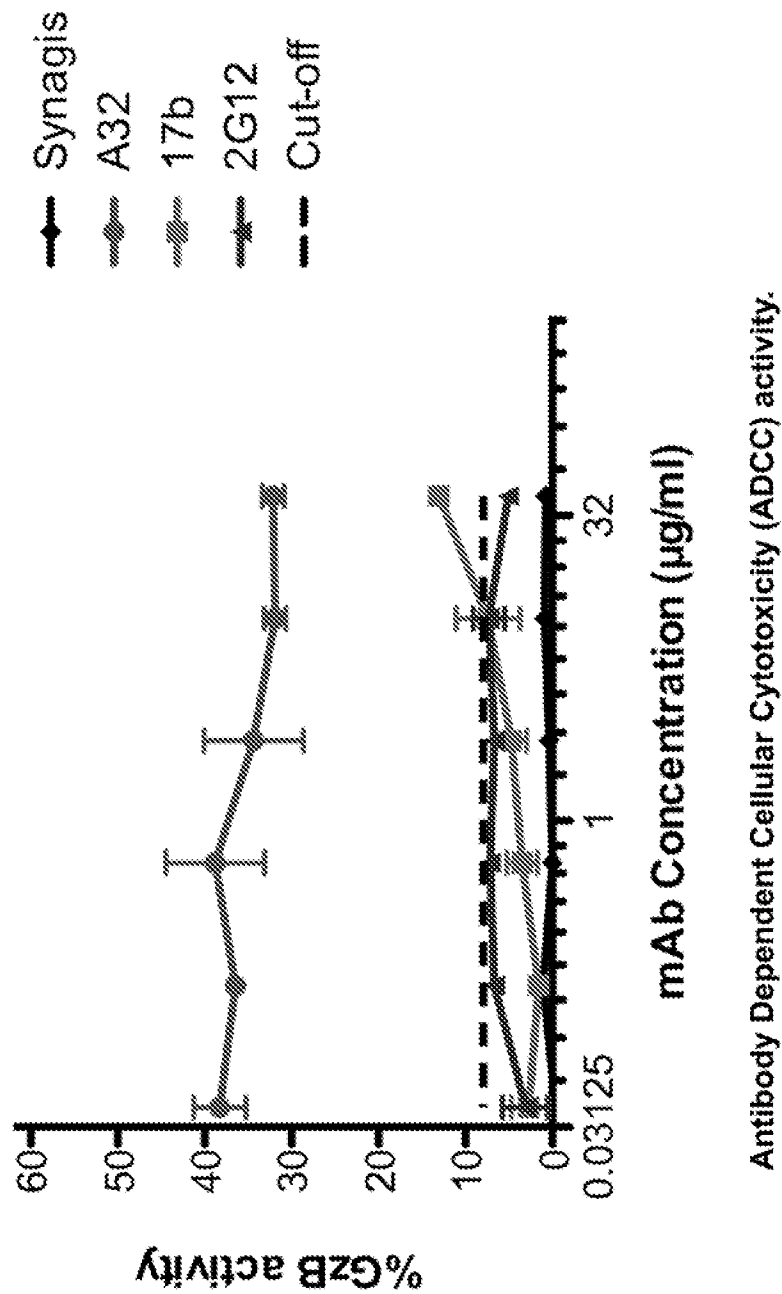
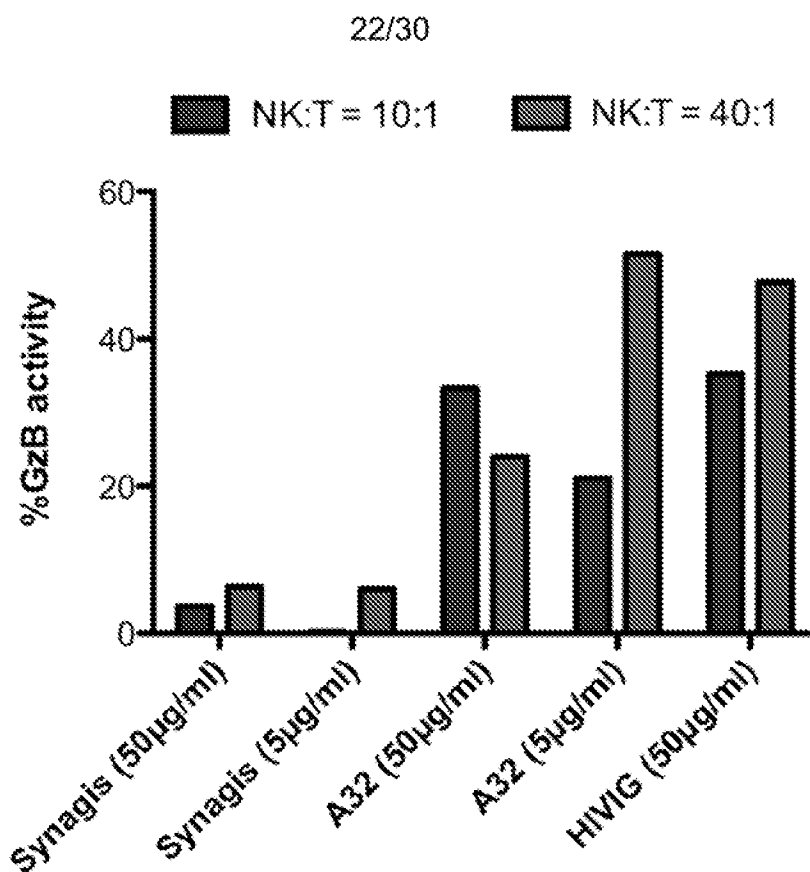


Figure 3 C. A1953_CEM.NKR_{CCR5}

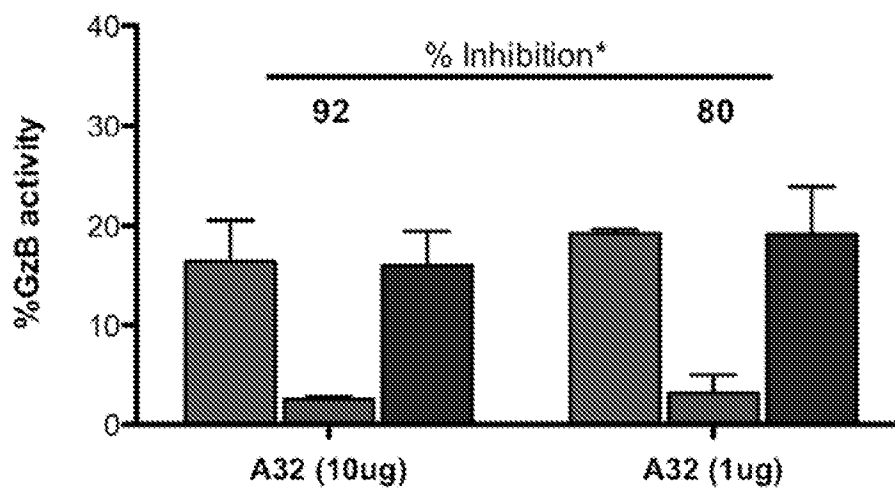




Antibody Dependent Cellular Cytotoxicity (ADCC) activity.

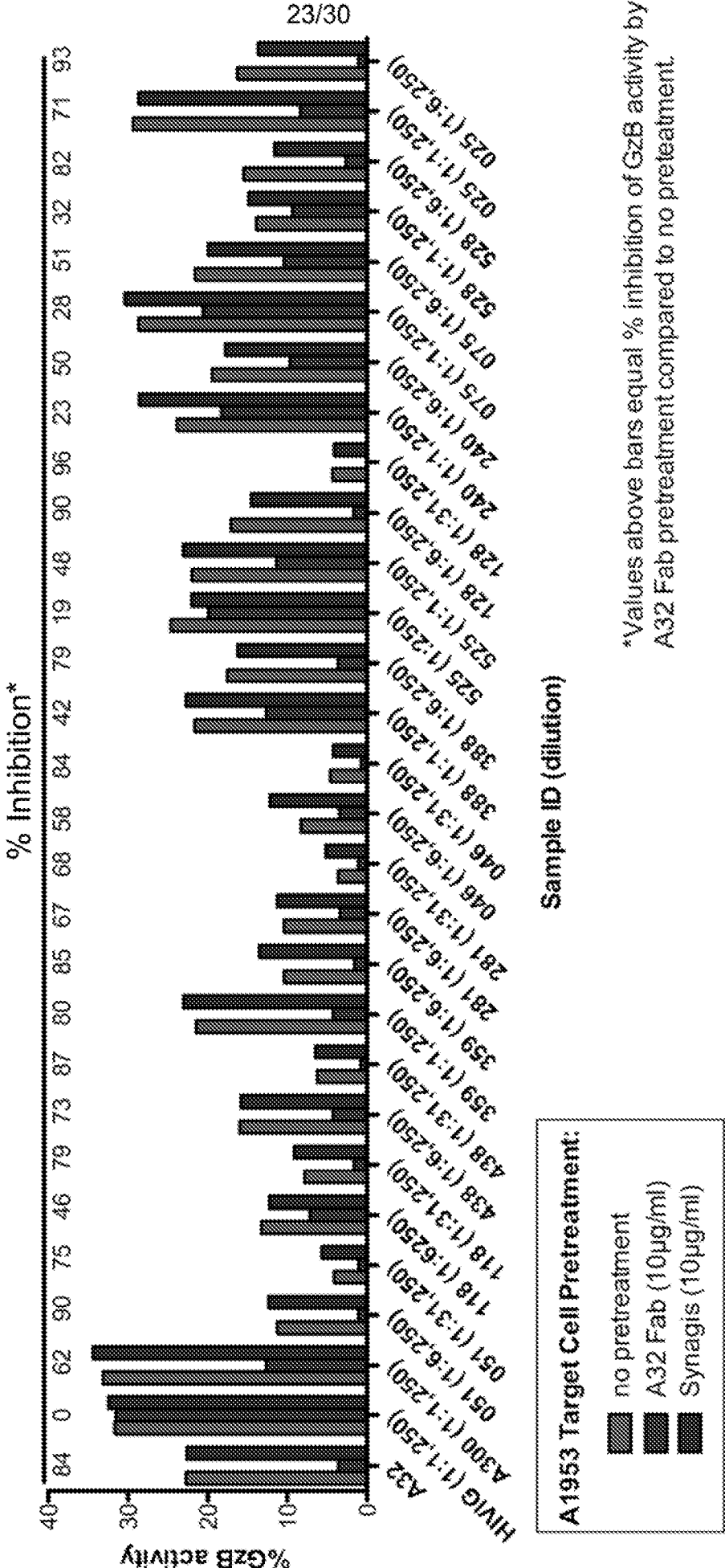
Figure 4

Figure 5 A. Inhibition of A32-mediated ADCC by A32 Fab



Inhibition of ADCC activity by A32 Fab fragment.

Figure 5 B. Inhibition of ADCC with A32 Fab



Inhibition of ADCC activity by A32 Fab fragment.

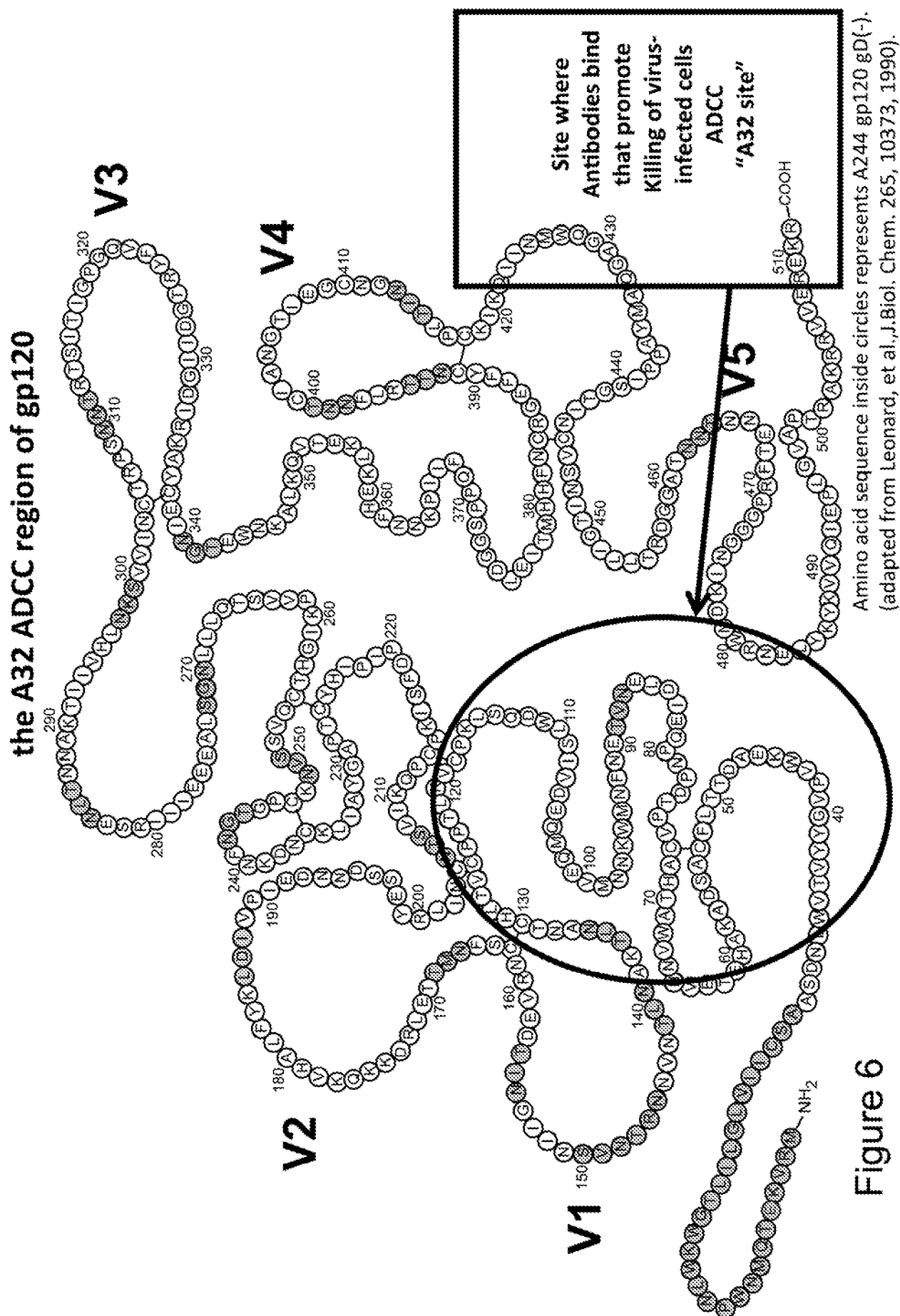
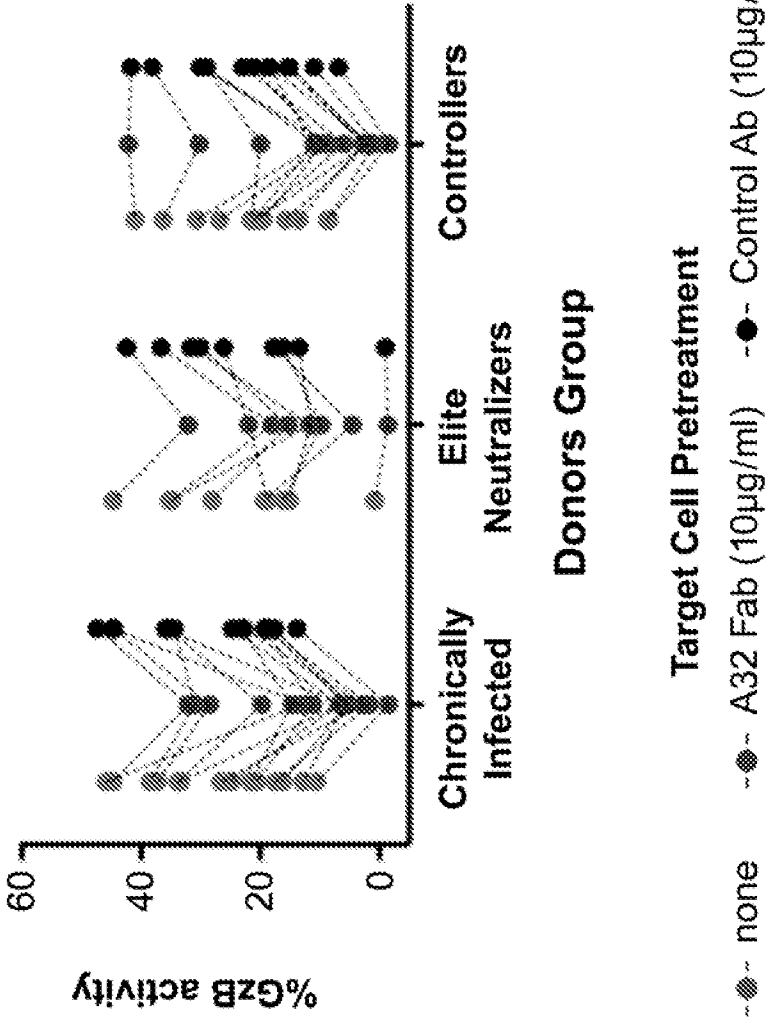


Figure 6

A32-like ADCC activity is present in a subset of Chronically infected (57%); Elite neutralizers (22%); and Controllers (69%)

Inhibition of ADCC activity by A32 Fab



A1953 Target Cells incubated with NK effector cells at E:T = 10:1

Figure 7

A32-like ADCC responses are responsible for the majority of the ADCC responses detectable in top 30 responders among the RV144 vaccine recipients using gp120 coated target cells

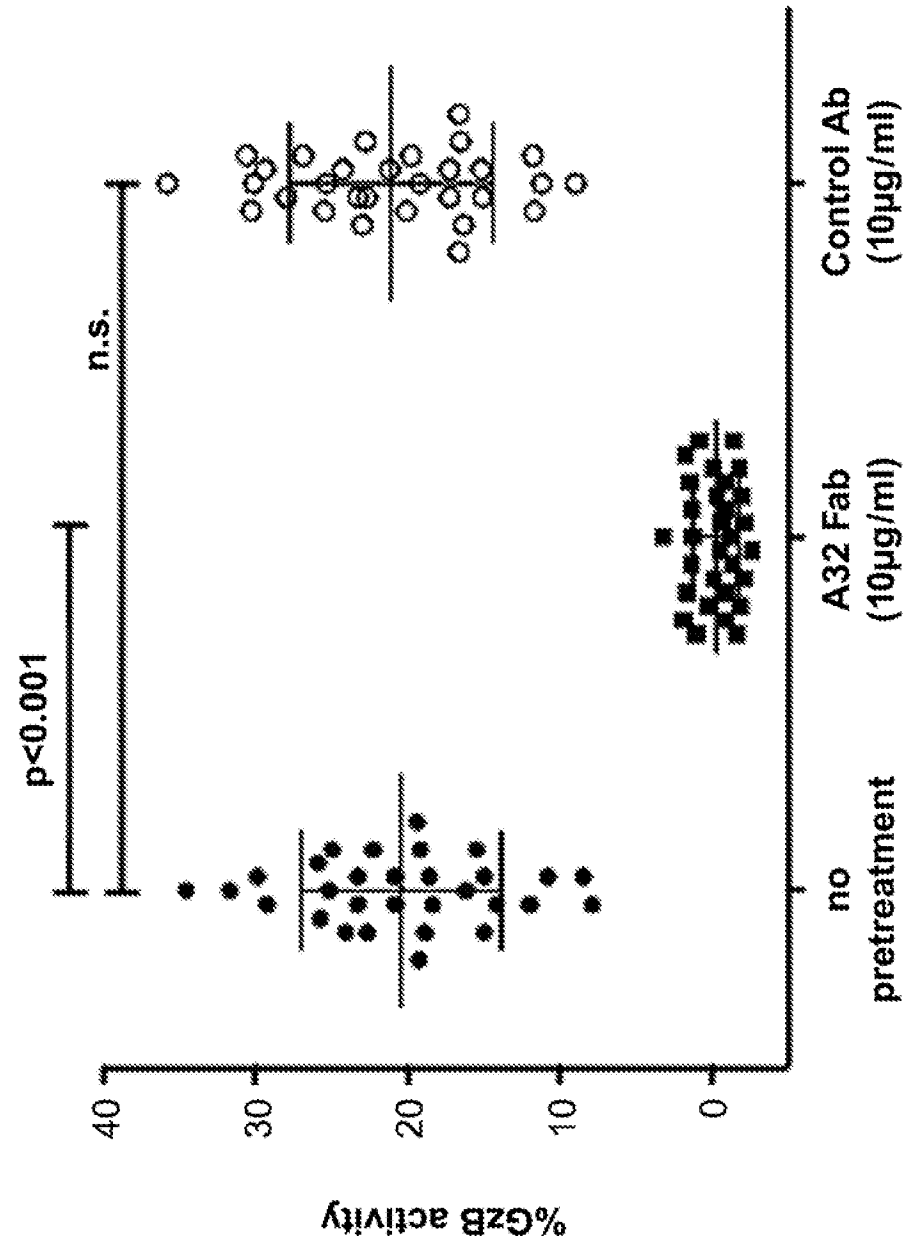


Figure 8

CM243gp120-coated Target Cell Pretreatment

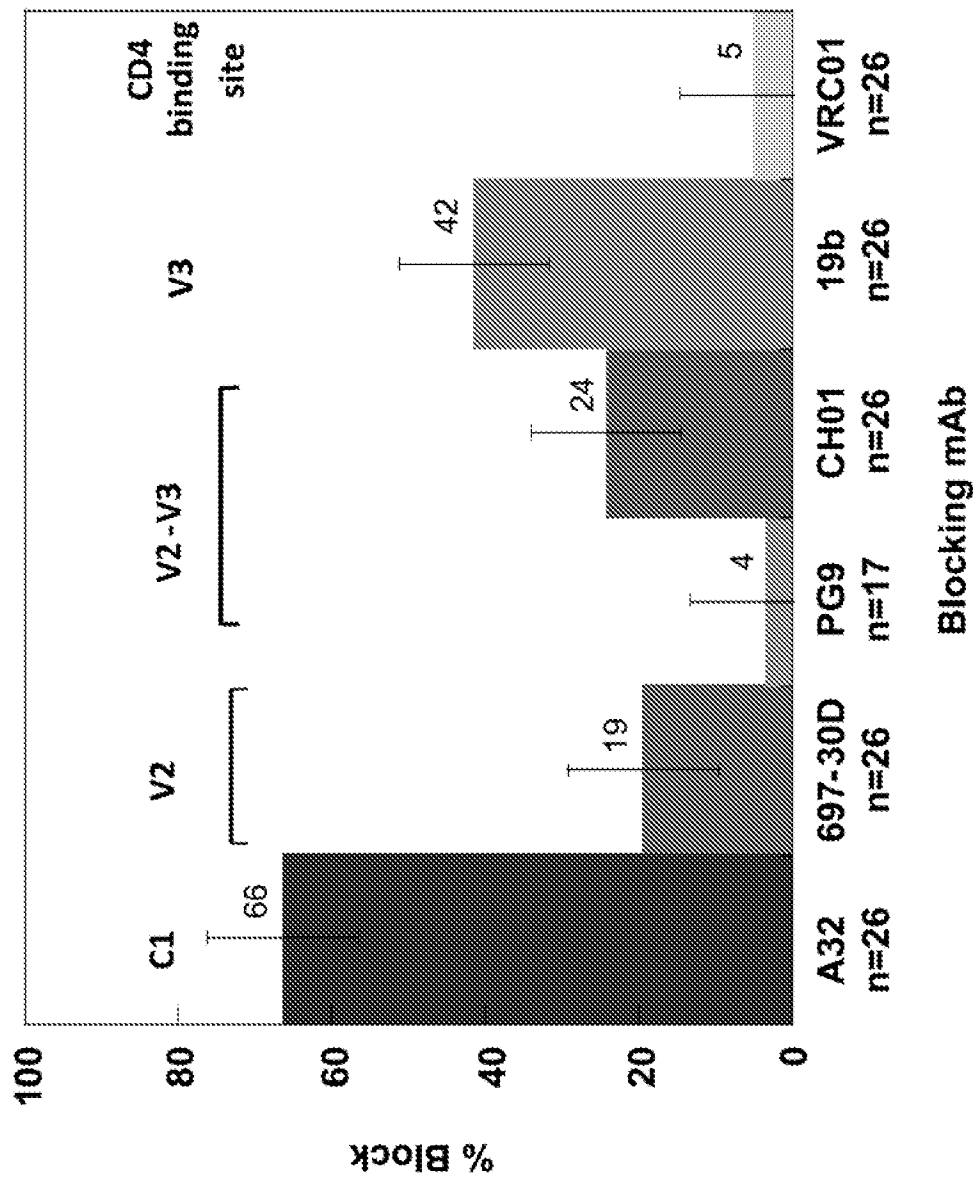


Figure 9

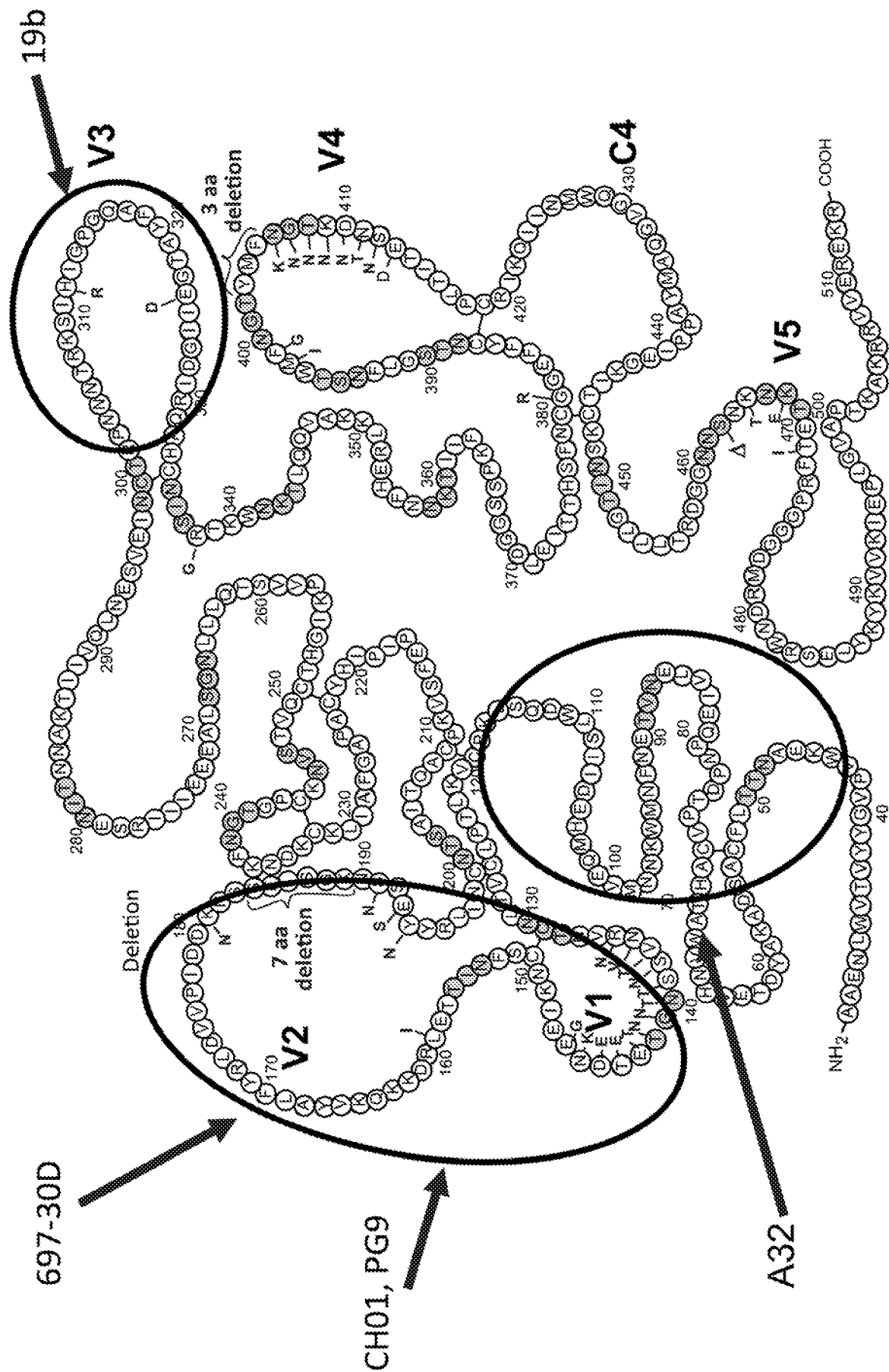


Figure 10

RV144 induced antibody responses on C1 (A32), V2 (697-30D), V3 (19b) and at or near the conformational-Quaternary V2-V3 epitope (CH01).

Quaternary mAb CH01 binding to A244 gp120 is abrogated by N160K mutation

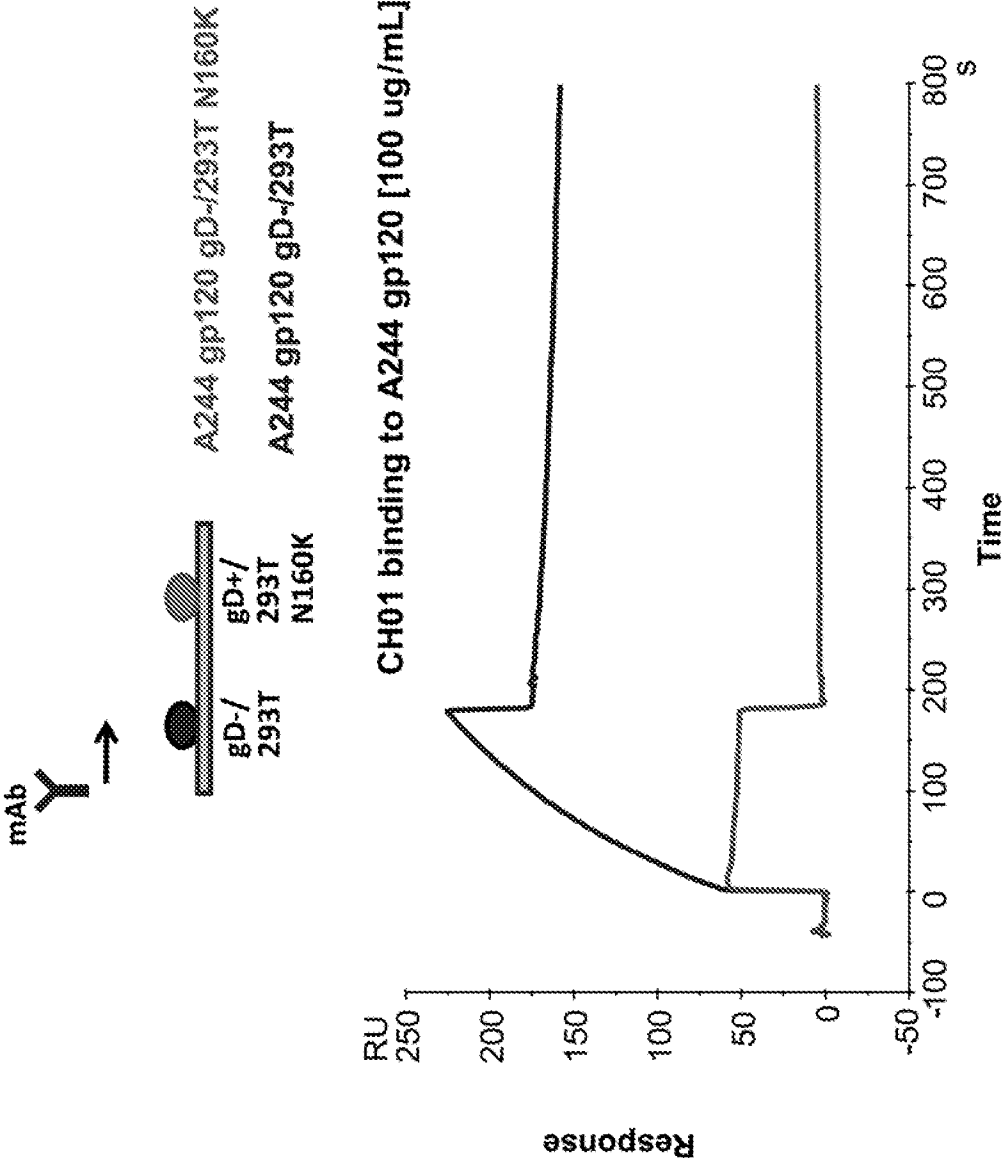


Figure 11

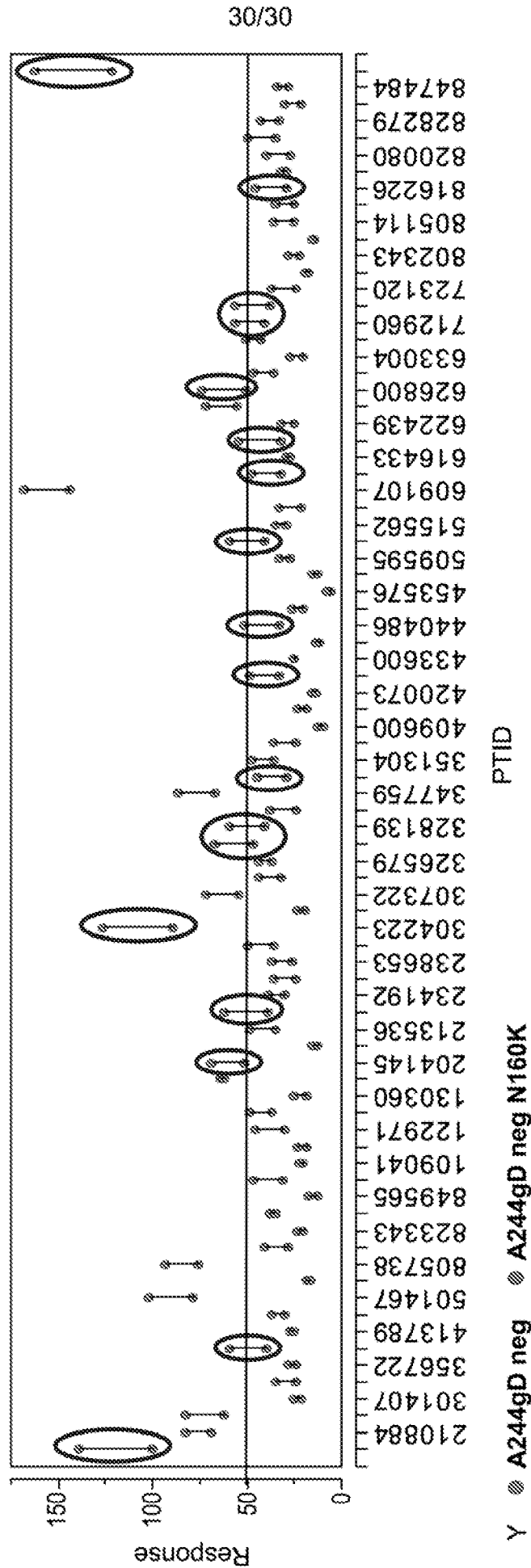


Figure 12