Supporting Information

A Universal CAR-NK Cell Targeting Various Epitopes of HIV-1 gp160

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I. MATERIALS AND REAGENTS

1. Anti-gp160 bNAbs

The following reagents were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: 1) Anti-HIV-1 gp120 Monoclonal (PG9) from IAVI;[1] 2) Anti-HIV-1 gp120 Monoclonal (PGT145) from IAVI;[2] 3) Anti-HIV-1 gp120 Monoclonal (PG16) from IAVI;[3] 4) Anti-HIV-1 gp120 Monoclonal (2G12) from Polymun Scientific; [4-8] 5) Anti-HIV-1 gp120 Monoclonal (PGT128) from IAVI;[2, 9] 6) 10-1074 MAb from Dr. Michel C. Nussenzweig;[10] 7) Anti-HIV-1 gp120 monoclonal (VRC01) from Dr. John Mascola (cat# 12033);[11] 8) Anti-HIV-1 gp120 Monoclonal (IgG1 b12) from Dr. Dennis Burton and Carlos Barbas;[12-15] 9) Anti-HIV-1 gp120 Monoclonal (3BNC117) from Dr. Michel C. Nussenzweig;[10, 16] 10) HIV-1 anti-gp41 mAb (10E8) from Dr. Mark Connors;[17] 11) Anti-HIV-1 gp41 Monoclonal (4E10) from Polymun Scientific;[18] 12) Anti-HIV-1 gp41 Monoclonal (2F5) from Polymun Scientific (cat# 1475);[4, 19, 20]

2. HIV gp160-expressing plasmids

The following reagents were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: 1) pConBgp160-opt (Cat#11402) from Dr. Beatrice Hahn;[21] and 2) pConCgp160-opt (Cat#11407) from Dr. Beatrice Hahn.[21, 22]

3. Lentivirus plasmids

The parental pFUW vector encoding an anti-CD19 CAR as well as the other three plasmids (pVSVG, pREV, and pRRE) for the third generation lentivirus system were kind gifts from Prof. Pin Wang (University of Southern California).

4. PCR primers

CD8 signal-*Bam*H I-F: 5'-ACGTGGATCCGCCACCATGGCTC-3'
αDNP-R: 5'-GGGTCTGGGTGCTGGAGTTGTAGTTGCAGAGACAGTGACCAGAGTCCC-3'
CD8 hinge-F: 5'- ACTACAACTCCAGCACCCAGACCC -3'
CD28-CD3ζ-*Eco*R I-R: 5'-AGTCGAATTCTCATCATCTTGGTGGCAGAG-3'
2G12-R: 5'-GGGTCTGGGTGCTGGAGTTGTAGTCCTCTTGATCTCCACCCTGGTGC-3'

5. Other reagents

HA-tag polyclonal rabbit antibody, F(ab')2-donkey anti-rabbit IgG (H+L) secondary antibody PE, Goat anti-human IgG Fc secondary antibody PE, donkey anti-goat IgG (H+L) secondary antibody

HRP, human IgG isotype control (Catalog No. 12000C), human anti-HLA-A2-APC (clone BB7.2), and human anti-CD56 (NCAM) APC were purchased from Thermo Fisher Scientific. The anti-human CD19 antibody (clone FMC63) was purchased from Novus Biologicals. Goat anti-dinitrophenol polyclonal antibody was purchased from Eagle Biosciences. CellTrace CFSE cell proliferation kit, CellTrace Blue Stain Reagent kit, LIVE/DEAD fixable aqua dead cell stain kit, Lipofectamine 2000, Geneticin (G418) solution, Magnisort streptavidin positive selection beads, Human T-Activator CD3/CD28 Dynabeads, recombinant human interleukin-2 (IL-2) (Catalog No. 34-8029-85), 8-16% Tris-Glycine SDS-PAGE gel, Gel Code Blue Stain Reagent, 2X sample loading buffer, 40 kDa MWCO 0.5 mL Zeba Spin Column, and IFN-γ human uncoated ELISA kit were purchased from Thermo Fisher Scientific. Transblot Turbo Mini PVDF Transfer Packs, Transblot Turbo System, and Clarity Western ECL Substrate were from BioRad. Retronectin and Lenti-X Concentrator were purchased from Takara Biosciences. N-(2,4-Dinitrophenyl)-6-aminocaproic acid N-succinimidyl ester (DNP-NHS ester) and β-mercaptoethanol were purchased from Sigma Aldrich. Amicron 100kDa MWCO concentrator was purchased from Millipore. RPMI, DMEM, fetal bovine serum, non-essential amino acids, sodium pyruvate, penicillin-streptomycin-glutamine were purchased from Thermo Fisher Scientific. FicoII-Paque Plus Reagent was purchased from GE Healthcare.

6. Cell culture

The NK-92MI cell line was purchased from ATCC. The HEK293T cell line was a gift from Prof. Pin Wang (USC). The HEK293 cell line was a gift of Prof. Wei-Chiang Shen (USC). Human buffy coats were purchased from Zen-Bio Inc (Research Triangle Park, NC). Human peripheral blood mononuclear cells (PBMC) were isolated from the buffy coat using FicoII-Paque density gradient centrifugation. NK-92MI cells were cultured in RPMI media supplemented with 20% fetal bovine serum and 0.1 mM non-essential amino acids, 1mM sodium pyruvate, 0.5mg/mL penicillin-streptomycin-glutamine, and 50μM β-mercaptoethanol. HEK293T cells were cultured in the DMEM medium supplemented with 10% fetal bovine serum and 0.5 mg/mL of penicillin-streptomycin-glutamine (PSG). HEK293 cells were also cultured in DMEM medium supplemented with 10% FBS, but without PSG in order to enable for selection with Geneticin. Human PBMCs were cultured in RPMI media supplemented with 10% fetal bovine serum and 0.1mM non-essential amino acids, 1mM sodium pyruvate, 0.5mg/mL penicillin-streptomycin-glytamine, and 50μM β-mercaptoethanol. PBMCs were activated using Human T-Activator CD3/CD28 Dynabeads and 50 IU/mL recombinant IL-2 cytokine for 3 days before lentivirus transduction with

anti-DNP CAR. Then primary human T-cells were further cultured using 50 IU/mL in complete RPMI medium before functional assays.

II. SUPPLEMENTAL METHODS

1. Construction of the traditional 2G12 CAR lentiviral vector pFUW-2G12-CD28-CD3ζ

The variable regions of anti-HIV-1 gp120 monoclonal antibody 2G12 were used to construct the 2G12 scFv [VH-$(GGGGS)_3$-VL]. An additional GGGGS linker was incorporated in between HA-tag and 2G12 scFv. The gene fragment consisting of the CD8 α signal peptide, HA-tag, GGGGS linker, and 2G12 scFv was synthesized by Integrated DNA Technologies (IDT, San Diego, CA). It was then amplified by PCR using primers CD8 signal-*Bam*H I-F and 2G12-R. This fragment was then linked together with CD8 hinge-CD28-CD3z (Ref. to Supplemental Methods II.1) by overlap PCR and digested with *Bam*H I and *Eco*R I. It was then ligated into the pFUW linear vector, and the ligation product was transformed into DH5α cells. Single colonies grown on LB-agar plates containing 100 μg/ml ampicillin were picked and grown in the LB liquid medium. The plasmid was purified, sequence verified, and then used to generate lentiviral particles by calcium-phosphate transfection of HEK293T cells.

2. Conjugation of PE to DNP

R-PE (1 mg/mL, Columbia Biosciences) was reacted with DNP-NHS ester at a molar ratio of 1:20 in 0.1 M sodium bicarbonate buffer, pH 8.3. After incubation at room temperature for 4 hours, the excess DNP-NHS was removed, and the buffer was changed into 1X PBS with 0.5% sodium azide utilizing a 40 kDa MWCO 0.5 mL Zeba Spin Column. The number of DNP moieties per R-PE molecule was determined based on measuring the absorbance at 280 nm (for R-PE) and 360 nm (for DNP) using the Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The average number of DNP moieties per R-PE was calculated using the following equation: [DNP]/[R-PE] =(A360/(ε_molar DNP (17,300 M^(-1) 〖 cm 〗 ^(-1))))/((A_280-(A_360×0.31))/(ε_molar R-PE (1,960,000 M^(-1) 〖cm〗^(-1)))).

3. Conjugation of bNAbs to DNP

All antibodies, including PG9, PGT145, PG16, 2G12, PGT128, 10-1074, VRC01, b12, 3BNC117, 10E8, 4E10, 2F5, and human IgG (1 mg/mL), were conjugated to DNP using the DNP-NHS ester (molar ratio 1:5) in 0.1M sodium bicarbonate buffer, pH 8.3. After overnight incubation at 4°C, the

excess DNP-NHS reagent was removed, and the buffer was changed into 1X PBS buffer containing 0.5% sodium azide, using a 40 kDa MWCO 0.5 mL Zeba Spin Column. The average number of DNP moieties per antibody was determined by measuring the absorbance at 280 nm (for antibodies) and 360 nm (for DNP) using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The final number of DNP moieties per antibody was calculated using the following equation: ([DNP])/([Antibody])=(A360/(ε_molar DNP (17,300 M^(-1) 〖cm〗^(-1))))/((A_280-(A_360×0.31))/(ε_molar Antibody (210,000 M^(-1) 〖cm〗^(-1)))).

4. Verification of DNP Conjugation to 2G12

DNP-conjugated and unconjugated antibodies 2G12 (2 μg/each) in 1X sample loading buffers were loaded onto pre-made 8-16% Tris-Glycine gels. After SDS-PAGE of two identical gels, the first gel was stained with GelCode Blue Stain Coomassie reagent to confirm that both reagents were loaded in equal amounts, and the second gel was subjected to western blotting analysis. To verify DNP conjugation by western blotting, proteins were first transferred to a PVDF membrane using the Transblot Turbo Blotting System. Then the membrane was blocked for 1 hour using 5% milk solution and incubated with a 1:500 dilution of the anti-DNP primary antibody overnight at 4°C. The membrane was washed three times using 1X Tris Buffered Saline with 0.05% Tween20 (1X TBST), incubated with a 1:5000 dilution of the donkey anti-goat IgG HRP secondary antibody for one hour at room temperature, and then washed three times using 1X TBST. Next, the membrane was incubated with an enhanced chemiluminescent (ECL) substrate and then imaged using the ChemiDoc Gel Imaging System (BioRad, Hercules, CA).

5. Flow cytometry-based antibody binding assay

The subtype B or C gp160-expressing HEK293 cells were stained with either VRC01, 3BNC117, 2G12, 10-1074, PG9, 10E8, or the isotype control at a 1:3 serial dilution from 300 nM to 0.045 nM. In the negative control, no antibody was added. To detect antibody binding, each sample was additionally stained using a PE-conjugated anti-human IgG secondary antibody (4 μg/mL). Stained cells were analyzed by flow cytometry using the LSRII (BD Biosciences, San Jose, CA), and the average fluorescence intensity per cell was analyzed using the FlowJo software (Ashland, OR).

6. Statistical analysis

All cell culture experiments were performed in triplicate, and data were presented as Mean ± SD. For verification of anti-DNP CAR-NK cell activation against subtype B gp160-expressing cells with

DNP-conjugated 2G12, two-way ANOVA was conducted using the 2G12 antibody, gp160-negative and positive cells as the independent variables. For assessing the optimal 2G12 adaptor molecule concentration for universal anti-DNP CAR activation, one-way ANOVA was conducted using the 2G12 antibody as the independent variable. For validation of anti-DNP CAR-NK cell activation against subtype B and subtype C gp160-expressing cells by IFN-γ production, one-way ANOVA was conducted using adaptor molecule types as the independent variable. For comparison of the different bNAbs in directing anti-DNP CAR cytotoxic activity against subtype B and subtype C gp160-expressing cells, a two-way ANOVA was conducted using antibody types and different E:T ratios as the independent variables. For comparison of the universal to the conventional 2G12 CAR-NK cell by flow cytometry-based cytotoxicity assays, a two-way ANOVA was conducted using CAR-NK cell types and different E:T ratios as the independent variables. For the comparison of the DNP-conjugated and unconjugated PG9 antibody in activating anti-DNP CAR-NK cells to produce IFN-γ, one-way ANOVA was conducted using the PG9 antibody type as the independent variable. For comparison of 10-1074 and 3BNC117 (either alone or in combination) in activating of universal CAR-NK cells, a one-way ANOVA was conducted using antibody type as the independent variable. For verification of anti-DNP CAR-T cell activation against subtype B gp160+ cells, a one-way ANOVA was conducted using antibody type as the independent variable. Both one-way and two-way ANOVA analyses were followed by either Tukey's or Dunnett's post hoc test for multiple comparisons. Significance was set at α=0.05. To draw the saturation curves for the bNAb binding assay to subtype B and C gp160-positive cells, a nonlinear regression curve fit, using the [Agonist] vs. response (three parameters) setting, was conducted. All statistical analyses were carried out using GraphPad Prism 8 (San Diego, CA).

III. FIGURES

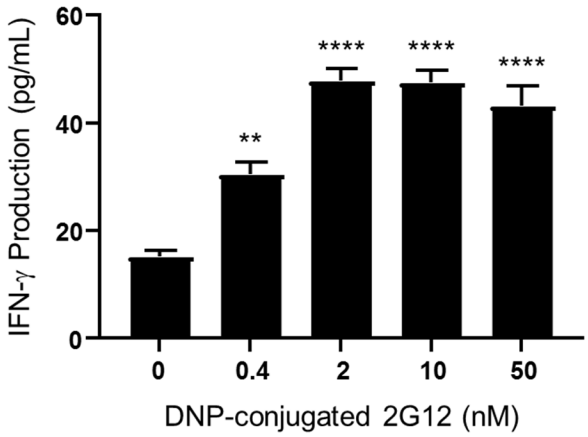

Figure S1. Identification of an optimal concentration of DNP-conjugated 2G12 for activating CAR-NK cells against subtype B gp160^{+} cells. The concentrations of IFN-γ in the culture supernatant were determined by ELISA. Data are presented as the mean ± SD of triplicate samples. Statistical significance is calculated by two-way ANOVA and Tukey's post-hoc analysis compared with the no antibody control. ** p<0.01, **** p<0.0001.

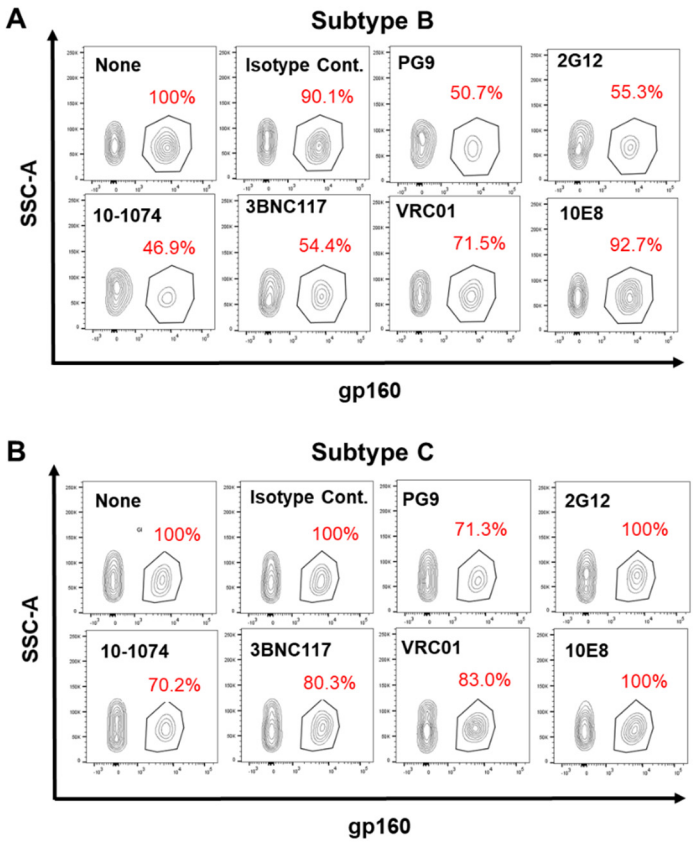

Figure S2. Universal CAR-NK cell-mediated killing of subtypes B and C gp160^{+} target cells. The subtype B or C gp160^{+} HEK293 cells were labelled with CFSE dye, while the gp160^{-} HEK293 cells were unlabelled. Anti-DNP CAR-NK cells were co-cultured with a 1:1 mixture of gp160^{+} and gp160^{-} cells at different E:T ratios (25:1, 5:1, and 1:1), with or without DNP-conjugated antibodies (2 nM). After an eight-hour incubation, cells were stained with an APC-conjugated anti-human HLA-A2 antibody and the aqua live/dead cell stain reagent, and then subjected to flow cytometric analysis. The gp160^{+} and gp160^{-} HEK293 cells were gated as HLA-A2-positive. Live HEK293 cells after co-culture with CAR-NK cells at the 25:1 E:T ratio are shown above. The percentages in red represent the remaining live gp160^{+} cells compared with the no antibody control (none).

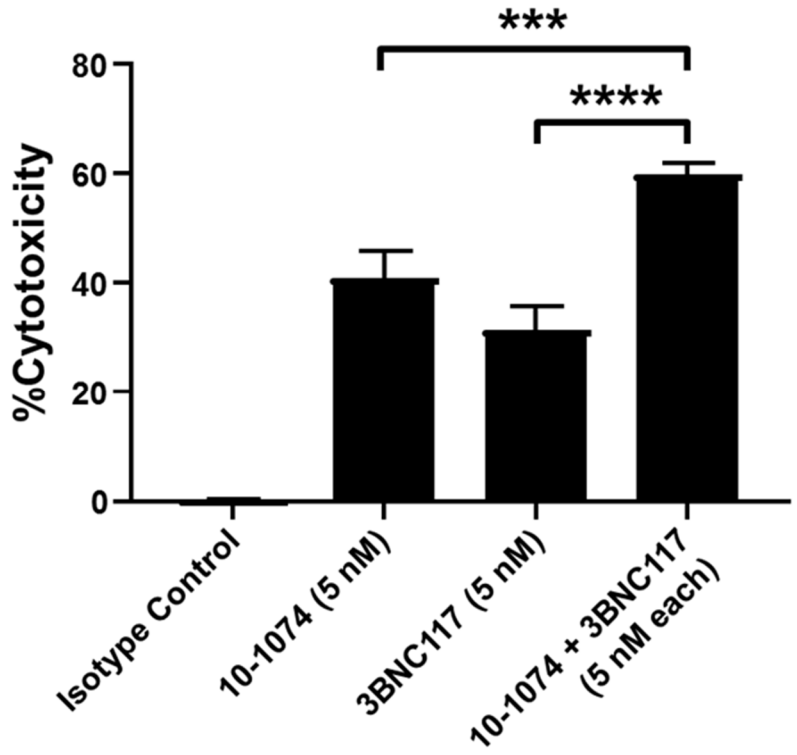

Figure S3. Targeting a mixture of subtype B and C gp160-expressing cells by CAR-NK cells supplemented with individual or combined bNAbs. The percentage of CAR-NK cell cytotoxicity was calculated as [(A-B)/Ax100], in which A and B were the numbers of viable $gp160^{+}$ cells (both subtypes B and C) after the cell co-culture was incubated without and with DNP-conjugated antibodies, respectively. Data are presented as the mean ± SD of triplicate samples. Statistical significance is calculated by two-way ANOVA and Tukey's post-hoc analysis.*** $p<0.001$,**** $p<0.0001$ vs. the isotype control.

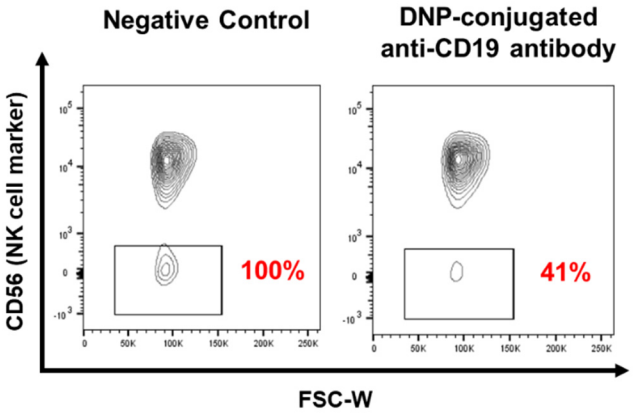

Figure S4. Redirection of the universal CAR-NK cell to target malignant B cells. Anti-DNP CAR-NK cells were co-cultured with REH cells at a 25:1 E:T ratio with or without a DNP-conjugated anti-CD19 antibody (clone FMC63, 50 nM). After overnight incubation, the percentages of remaining REH cells were determined by flow cytometry. CD56 is a cell surface marker for NK cells.

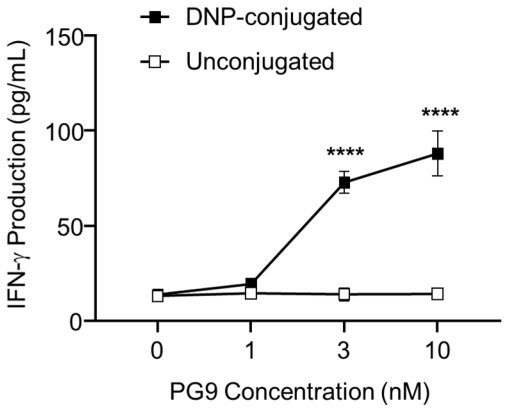

Figure S5. The unconjugated antibody PG9 cannot redirect anti-DNP CAR-NK cells to target gp160^{+} cells. Anti-DNP CAR-NK cells were co-cultured with the subtype B gp160-expressing cells in the presence of DNP-conjugated and unconjugated antibody PG9. The concentrations of IFN-γ in the culture supernatant were determined by ELISA. Data are presented as the mean ± SD of triplicate samples. Statistical significance is calculated by two-way ANOVA and Tukey's post-hoc analysis compared with the unconjugated PG9. **** p<0.0001.

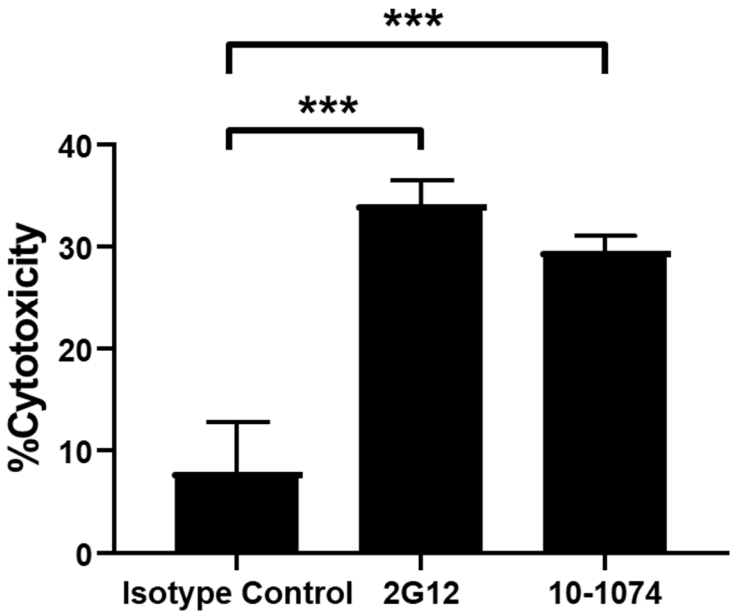

Figure S6. Universal anti-DNP CAR-T cells can also kill subtype B gp160+ cells. Primary human anti-DNP CAR-T cells were co-cultured with CellTrace Blue-labeled HEK293 gp160+ Subtype B cells at a 1:1 E:T ratio in the presence of 10 nM of DNP-conjugated bNAbs or isotype control. After a 48-hour incubation at 37˚C, cells were collected and stained with a FITC-conjugated anti-human CD3 antibody and an aqua live/dead cell stain reagent, followed by flow cytometric analysis. The percentage of CAR-T cell cytotoxicity was calculated as [(A-B)/Ax100], in which A and B were the numbers of viable $gp160^{+}$ cells after the cell co-culture was incubated without and with the addition of DNP-conjugated antibodies, respectively. Data are presented as the mean ± SD of triplicate samples. Statistical significance is calculated by one-way ANOVA and Dunnett's post-hoc analysis compared to the isotype control. ** p<0.01, *** p<0.001. n.s.: not significant.

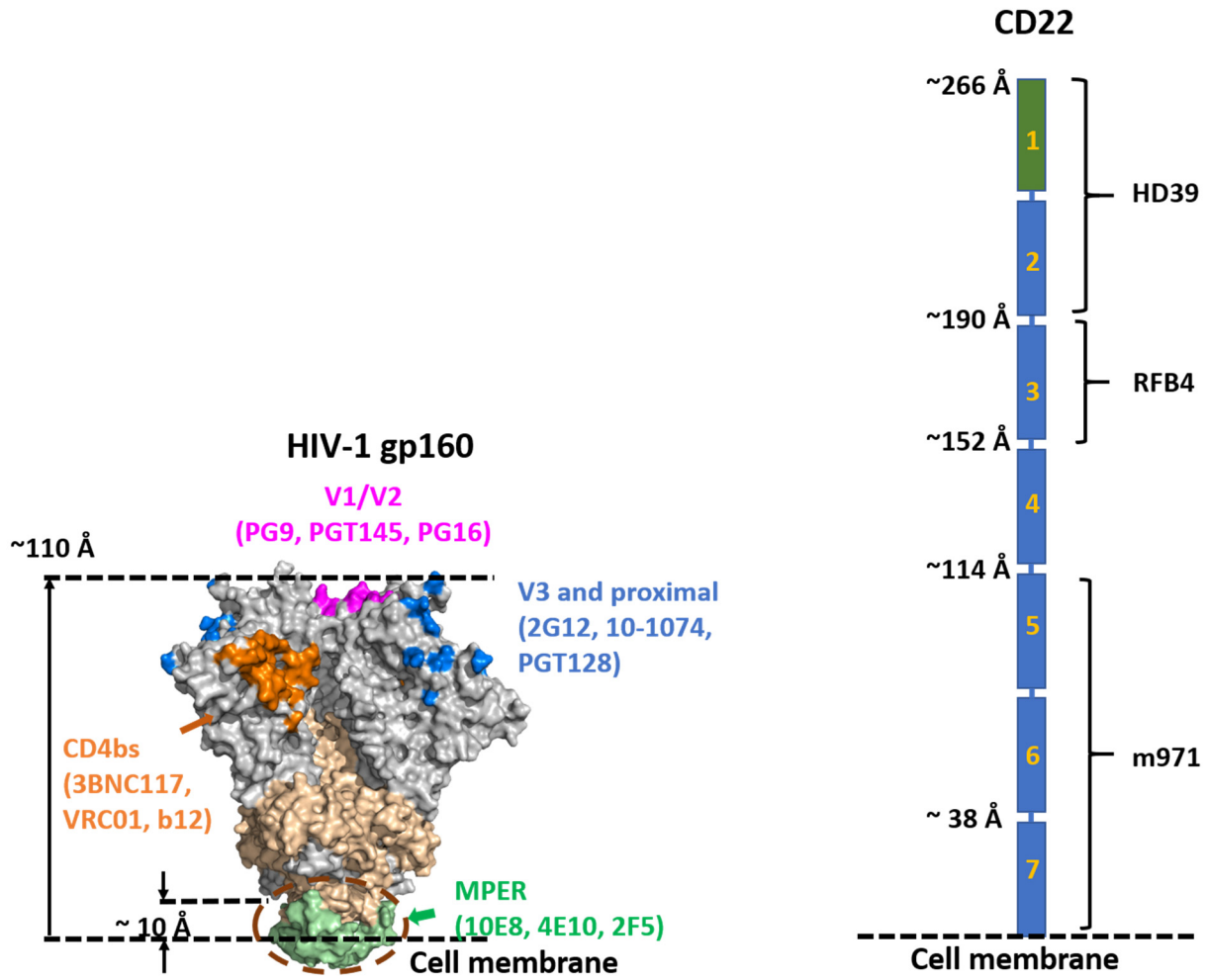

Figure S7. Comparison of the distances of different gp160 and CD22 epitopes to the cell membrane. The extracellular domain of CD22 consists of seven Ig-like domains. Based on the structure of CD22 (PDB ID: 5VKJ), the average length of each Ig-like domain is estimated to be about 38 Å.

IV. REFERENCES

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