1 **SUPPLEMENTAL METHODS**

2

3 **Primary cells**

4 PBMC were obtained by leukapheresis. NK cells and CD4+ T lymphocytes were purified
5 from resting PBMCs by negative selection using immunomagnetic beads per the
6 manufacturer's instructions (StemCell Technologies, Vancouver, BC). NK cells were
7 cultured overnight in RPMI 1640 complete medium supplemented with 20% fetal bovine
8 serum and 100 μg/mL penicillin-streptomycin before use. CD4+ T lymphocytes were
9 activated with phytohemagglutinin-L (10 μg/ mL) for 48 hours and then maintained in
10 RPMI 1640 complete medium supplemented with rIL-2 (100 U/mL).

11

12 **Antibodies**

13 The following Abs were used as first Ab for cell surface staining: 1 μg/mL mouse anti-
14 CD4 mAb OKT4 (14-0048-82; eBiosciences), 5 μg/mL human anti-HIV-1 Env mAbs
15 A32, PGT126 (kindly provided by the International AIDS Vaccine Initiative), 3BNC117
16 (kindly provided by M.C. Nussenzweig, The Rockefeller University, New York, NY), 1
17 μg/mL of either Alexa Fluor 647-conjugated goat anti-mouse or goat anti-human Abs
18 (Thermo Fisher Scientific) were used as secondary Abs. The following Abs were used
19 for the NK cell activation assay: APC-conjugated anti-CD107a (BD Biosciences, H4A3),
20 BV421-conjugated anti-CD3 (Biolegend, UCHT1), PE-conjugated anti-CD56 (BD
21 Biosciences, NCAM16.2) and PE-Cy7-conjugated anti-IFNγ (BD Biosciences, B27). The
22 following Abs were used for RNA-flow analysis: BUV395-conjugated anti-CD3 (BD
23 Biosciences, UCHT1), PE-Cy7-conjugated anti-CD4 (BD Biosciences, RPA-T4) BV510-

24 conjugated anti-CD8 (Biolegend, SK1), BV510-conjugated anti-CD14 (Biolegend,
25 M5E2) BV510-conjugated anti-CD19 (Biolegend, H1B19) and PE-conjugated anti-p24
26 (Beckman Coulter/Immunotech, KC57), while AquaVivid (Thermo Fisher Scientific)
27 was used as viability dye.

28

29 **ADCC measurements**

30 **FACS-based assay**

31 Measurement of ADCC using the FACS-based assay was performed at 48h post-
32 infection as previously described (1-3). Briefly, infected primary CD4+ T cells were
33 stained with viability (AquaVivid; Thermo Fisher Scientific) and cellular (cell
34 proliferation dye eFluor670; eBioscience) markers and used as target cells. Autologous
35 PBMC effectors cells, stained with another cellular marker (cell proliferation dye
36 eFluor450; eBioscience), were added at an effector: target ratio of 10:1 in 96-well V-
37 bottom plates (Corning, Corning, NY). A 1:1,000 final dilution of sera or 5 μg/ml of
38 ADCC-mediating mAbs were added to appropriate wells and cells were incubated for 15
39 min at room temperature. The plates were subsequently centrifuged for 1 min at 300 g,
40 and incubated at 37°C, 5% CO_2 for 5 to 6 h before being fixed in a 2% PBS-
41 formaldehyde solution containing $5x10^4$ flow cytometry particles/ml (AccuCount blank
42 particles; 5.3 m; Spherotech). Samples were analyzed on an LSRII cytometer (BD
43 Biosciences) and acquisition was set to acquire 1,000 flow cytometry particles, which
44 allows the calculation of relative cell counts (2). Data analysis was performed using
45 FlowJo vX.0.7 (Tree Star). The percentage of ADCC was calculated with the following
46 formula: (relative count of GFP+ cells in targets plus effectors) - (relative count of GFP+

47 cells in targets plus effectors plus Abs or sera) / (relative count of GFP+ cells in targets)
48 by gating infected live target cells. For cells infected with HIV-1 primary isolates,
49 infected cells were identified by intracellular staining for HIV-1 p24. In that context, the
50 percentage of ADCC was calculated with the following formula: (% of p24+ cells in
51 Targets plus Effectors) − (% of p24+ cells in Targets plus Effectors plus sera) / (% of
52 p24+ cells in Targets) by gating on infected lived target cells.

53

54 **Granzyme B assay**

55 Measurement of ADCC response using the Granzyme B assay was performed 48h
56 post-infection according to the manufacturer's instructions (OncoImmunin, Gaithersburg,
57 MD) and as previously described (4, 5). Briefly, infected primary CD4+ T cells were
58 stained with a fluorescent target cell marker (TFL4; OncoImmunin, Gaithersburg, MD)
59 and a viability marker (NFL1; OncoImmunin or AquaVivid; Thermo Fisher Scientific).
60 Rested autologous purified NK cells were used as effector cells. Target and effector cells
61 were counted and adjusted to reach a final effector: target ratio of 10:1. Twenty-five μl of
62 each effector and target cell suspension and 75 μl of Granzyme B substrate
63 (OncoImmunin) was dispensed into each well of a 96-well V-bottom plate (Corning). A
64 1:1,000 final dilution of sera or 5 μg/ml or ADCC-mediating Abs were added to
65 appropriate wells and cells were incubated for 15 min at room temperature. The plates
66 were subsequently centrifuged for 1 min at 300 g, and incubated for 1 h at 37°C and 5%
67 CO_2. After two washes, cells were acquired on an LSRII cytometer (BD Biosciences)
68 and data analysis was performed using FlowJo vX.0.7 (Tree Star). The percentage of Ab
69 or sera-induced Granzyme B activity was calculated with the following formula:

70 (Percentage of Granzyme B+ target cells in targets plus effectors plus Abs or sera) -
71 (Percentage of Granzyme B+ target cells in targets plus effectors) per the gating strategy
72 presented in Fig S5.
73

74 **NK cell activation assay**

75 Measurement of ADCC response using the NK cell activation assay was
76 performed using a modified version of a previously described protocol (6). Briefly,
77 primary CD4+ T cells infected for 48h were co-cultured with autologous PBMC at an
78 effector: target ratio of 5:1 in the presence of a 1:1,000 final dilution of sera or 5 μg/ml or
79 ADCC-mediating Abs, anti-CD107a, Brefeldin A (Sigma) (5μg/ml) and Monensin (BD
80 Biosciences) (6μg/ml) for 5h at 37°C. Control conditions included incubation of PBMC
81 alone or incubation of PBMC with mock cells in the presence of sera or ADCC-
82 mediating Abs. After incubation cells were surface stained with anti-CD3 and anti-CD56
83 Abs. Next, cells were fixed and permeabilized using the Cytofix/Cytoperm Fixation/
84 Permeabilization Kit (BD Biosciences) and stained with anti-IFNγ Ab. Samples were
85 analyzed on an LSRII cytometer (BD Biosciences) and data analysis was performed
86 using FlowJo vX.0.7 (Tree Star). The percentage of Ab or sera-induced NK cell
87 activation was calculated with the following formula: (Percentage of NK cells (CD3-
88 CD56+) positive for CD107a and/or IFNγ in targets plus effectors plus Abs or sera) -
89 (Percentage of NK cells (CD3-CD56+) positive for CD107a and/or IFNγ in targets plus
90 effectors) according to the gating strategy presented in Fig S5.
91

92 **Luciferase assay**

93 Measurement of ADCC responses using the Luciferase assay was performed as
94 previously described (7). Briefly, CEM.NKR-CCR5-sLTR-Luc target cells, which
95 express luciferase (Luc) under the control of a Tat-inducible promoter, were infected and
96 used as target cells at 48h post-infection. Primary human PBMCs or NK cell line
97 expressing human CD16 were used as effector cells. Target and effector cells were co-
98 cultured for 6-8h, in triplicate, at an effector: target ratio of 10:1 in the presence of
99 different concentrations of Abs (0.0024, 0.0098, 0.0390, 0.1563, 0.6250, 2.5, 10 or 40
100 μg/ml) or different dilution of human sera (1:100, 1:400, 1:1600, 1:6400, 1:25600,
101 1:102400, 1:409600 or 1:1638400). The dose-dependent loss of Luc activity was
102 measured as an indication of sera or Ab-mediated killing of productively-infected cells.
103 Infected target cells incubated with effector cells in the absence of sera or Ab were used
104 to measure maximal Luc activity, and uninfected target cells cultured with effector cells
105 were used to determine background Luc activity. Area under the curve (AUC) values for
106 ADCC were calculated as previously described (7). For experiments involving CH77 wt
107 and N-U- viruses, the *vif* gene was deleted by site-directed mutagenesis. The nucleotides
108 62 and 63 in *vif* were deleted, causing a frame-shift that resulted in an early stop codon
109 (W21*) while preserving the *pol* open reading frame.
110
111 **RFADCC assay**
112 CEM.NKR-CCR5-sLTR-Luc target cells were coated or not with 1 μg/ml of
113 recombinant gp120 protein derived from the HIV-1 YU2 strain for 30 minutes at 37°C
114 +5% CO_2. Cells were then double stained with PKH26 red fluorescent cell linker
115 (Sigma-Aldrich, USA) and the cellular proliferation marker eFluor670 (eBioscience) as

116 previously described (2, 8, 9) Cells were then dispensed together with different
117 concentration of Abs (0.008, 0.04, 0.2, 1 and 5 μg/ml) or different dilutions of sera
118 (1:100, 1:400, 1:1600, 1:6400 and 1:25600). After 15 min at room temperature, effector
119 cells (Rested primary PBMCs) were added at an effector: target ratio of 50:1. Plates were
120 centrifuged for 1 min at 300 g and incubated for 4 h at 37°C. Cells were washed, fixed
121 and acquired on an LSRII cytometer (BD Biosciences). Data analysis was performed
122 using FlowJo vX.0.7 (Tree Star). The percentage of cytotoxicity was calculated with the
123 following formula: (Percentage of cells that remained PKH26+ but that had lost the
124 proliferation dye (eFluor670-) in target cells coated with recombinant gp120 plus
125 effectors plus Abs or sera) - (Percentage of cells that remained PKH26+ but that had lost
126 the proliferation dye (eFluor670-) in target cells plus effectors plus Abs or sera)
127 according to the gating strategy presented in Fig S6.
128
129

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174 **SUPPLEMENTAL FIGURE LEGEND**

175

176 **Fig S1. Level of cell-surface CD4 on infected and uninfected cells.** Cell-surface
177 staining of primary CD4+ T cells mock-infected (Mock) or infected with the NL4.3 ADA
178 GFP virus, either wild-type (HIV WT) or defective for Nef and Vpu expression (HIV N-
179 U-) with the anti-CD4 OKT4 Ab (1μg/ml) (A) Dot plots depicting representative
180 staining. (B) Mean Fluorescence Intensity (MFI) obtained for at least 5 different
181 experiments. Error bars indicate means ± standard errors of the means. Statistical
182 significance was tested using Ordinary one-way ANOVA. (*** $p<0.001$, ns: non-
183 significant).

184

185 **Fig S2. Recognition of primary CD4+ T cells infected with the transmitted/founder**
186 **virus CH77.** Primary CD4+ T cells were mock-infected or infected with the
187 transmitted/founder virus CH77, either wild-type (HIV WT) or defective for Nef and Vpu
188 expression (HIV N-U-). Forty-eight hours post-infection, cells were stained with A32,
189 PGT126, 3BNC117 (5 μg/ml) or sera from 10 HIV-1-infected individuals (HIV+ sera) or
190 5 uninfected individuals (HIV- sera) (1:1000 dilution), followed with appropriate
191 secondary Abs. Shown in (A) are the mean fluorescence intensities (MFI) obtained for at
192 least 5 independent stains with the different Abs, with 10 HIV+ or 5 HIV- sera on cells
193 infected with WT virus. Graphs shown in (B) represent the MFI obtained for 5
194 independent stains with A32, with 10 HIV+ sera or 5 HIV- sera on cells infected with
195 WT and N-U- virus. Error bars indicate means ± standard errors of the means. Statistical

significance was tested using (A) Ordinary one-way ANOVA, (B) unpaired t test or Mann-Whitney test (* p<0.05, (** p<0.01, **** p<0.0001, ns: non-significant).

Fig S3. ADCC responses detected by the Luciferase assays. Representation of the ADCC responses detected by the Luc assay (presented in Fig 2) as the loss of luciferase signal (RLU). (A) ADCC responses mediated by A32, PGT126 and 3BNC117 (0.0024, 0.0098, 0.0390, 0.1563, 0.6250, 2.5 and 10 μg/ml) against cells infected with NL4.3 ADA GFP WT. (B) ADCC responses mediated by A32 (0.0024, 0.0098, 0.0390, 0.1563, 0.6250, 2.5 and 10 μg/ml), HIV+ or HIV- sera (1:100, 1:400, 1:1600, 1:6400, 1:25600 and 1:102400) against cells infected with NL4.3 ADA GFP WT (HIV WT) or defective for Nef and Vpu expression (HIV N-U-). Error bars indicate means ± standard errors of the means.

Fig S4. ADCC responses detected with the FACS-based and Luciferase assays against cells infected with the transmitted/founder virus CH77. (A-C) Primary CD4+ T cells or (D-F) CEM.NKR-CCR5-sLTR-Luc cells infected with the transmitted/founder virus CH77, either wild-type (HIV WT) (depicted in gray) or defective for Nef and Vpu expression (HIV N-U-) (depicted in black) were used as target cells with the (A-C) FACS-based infected cell elimination assay or (D-F) the Luciferase assays. Shown in (A,D) are ADCC responses mediated by A32, PGT126 and 3BNC117 against cells infected with WT virus, and in (B,C,E,F) ADCC responses mediated by (B,E) A32 or (C,F) HIV+ and HIV- sera against cells infected with WT or N-U- virus. (A-C) All graphs shown represent ADCC responses obtained from at least 5 independent

experiments. (D-F) ADCC responses presented as loss of luciferase signal (RLU) in triplicate. For the FACS-based assay, mAbs were used at 5μg/ml and human sera at a 1:1000 dilution. For the Luciferase assay, mAbs were used at 0.0024, 0.0098, 0.0390, 0.1563, 0.6250, 2.5, 10 and 40 μg/ml and human sera at a dilution of 1:100, 1:400, 1:1600, 1:6400, 1:25600, 1:102400, 1:409600 and 1:1638400. Error bars indicate means ± standard errors of the means. Statistical significance was tested using unpaired t test (*** $p<0.001$, **** $p<0.0001$, ns: non-significant).

Fig S5. Gating strategy used for the NK cell activation and Granzyme B assays. Gating strategy and dot plots depicting a representative detection of (A) NK cell activation and (B) granzyme B activity in the context of cells infected with NL4.3 ADA GFP virus, either WT (HIV WT) or defective for Nef and Vpu expression (HIV N-U-) in the presence or absence of A32.

Fig S6. Gating strategy used for the RFADCC assays. Gating strategy and dot plots depicting representative detection of responses mediated by A32 against target cells coated or not with recombinant gp120 using the RFADCC assay.

Fig S7. The vast majority of uninfected bystander CD4+ T cells remain uninfected after 5 days in culture. Primary CD4+ T cells were infected with the NL4.3 ADA GFP WT. Forty-eight hours post-infection, the GFP- cell population was sorted by flow cytometry and kept in culture for 5 days. The vast majority of cells (>96%) remained

241 negative for GFP after 5 days of culture. Error bars indicate means ± standard errors of
242 the means for 3 independent experiments.
243
244 **Fig S8. Characterization of cells infected with the X4-tropic NL4.3 virus by RNA-**
245 **Flow FISH method.** Quantification of the percentage of cells positive for A32 binding
246 and *Gag-Pol* mRNA based on CD4 and p24 levels among primary CD4+ T cells infected
247 with the X4-tropic NL4.3 WT virus. Error bars indicate means ± standard errors of the
248 means of at least 4 independent experiments.