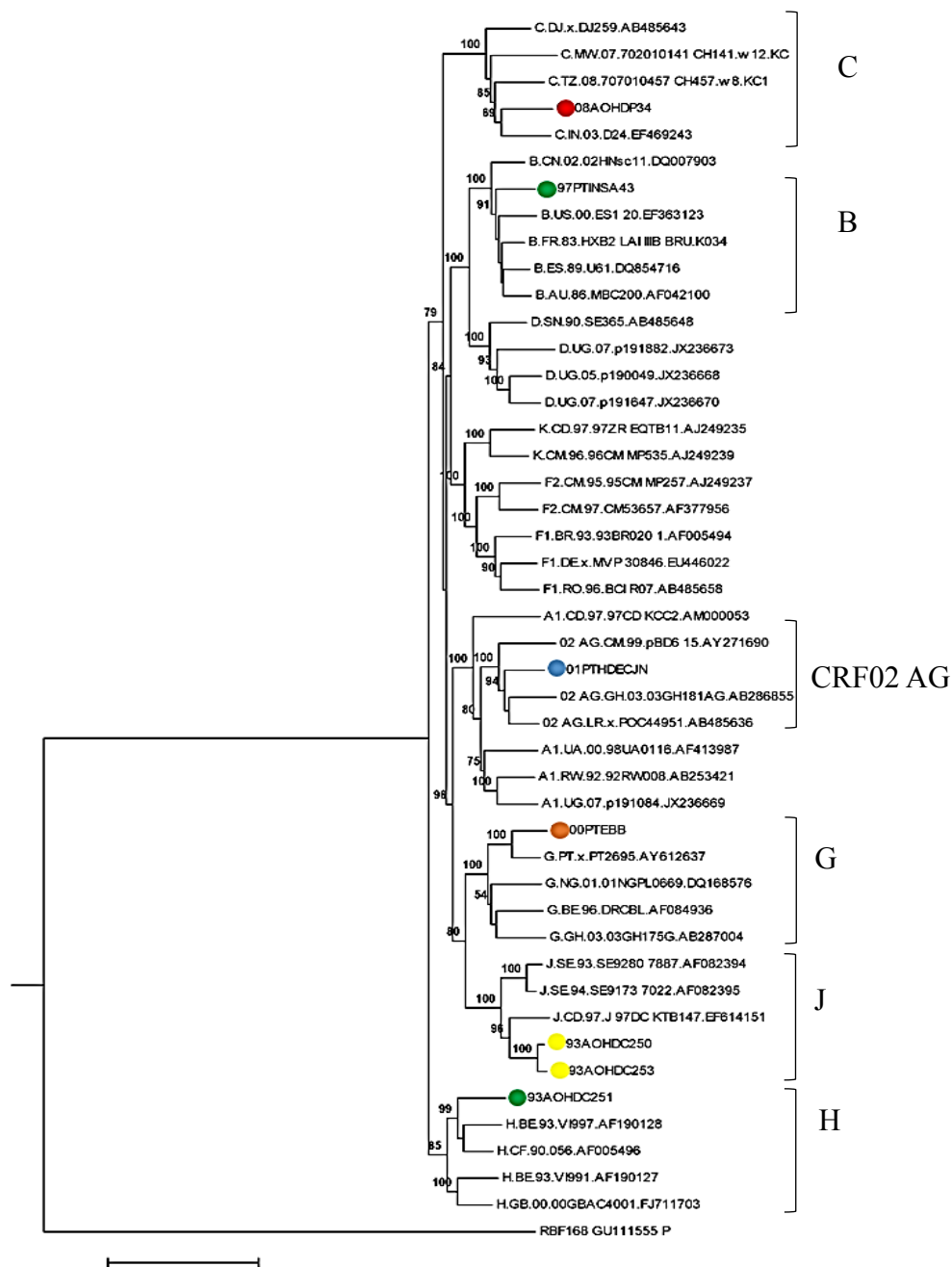




**Figure S1 - Phylogenetic analysis of HIV-1 *env* genes used in this study.** The maximum likelihood phylogenetic trees were constructed with alignments of reference sequences representative of all HIV-1 group M subtypes and the *env* gene sequences produced in this study (represented with colored dots). Sequence RBF168 GU111555 belonging to HIV-1 group P was used as outgroup. Only bootstrap values above 70% are shown.

gp120/gp41 interface (PGT151)

```

      *      20      *      40      *      60      *      80      *      100      *
HXB2      : MRVK---EKYQHLWRWGWRTMLLGLMLICSATEKLWVTVYYGVPVWKEATTLFCASDAKAYDTVEVHNVWATHACVPTDPNPQEVVLNVVTENFNMWKNDMVEQMHEDIISLW : 112
JR FL      : ....GIRKN....---..G..L...IIV...V.....G....K.....N....Q..... : 111
93A0HDC250 : .K.METQTSWKS.---...L.IF..F...K...Q.....K.....S.S..G..I.....I..E.....I.E.....V. : 111
08A034HDP  : ...MGIMRNC.QW---.I..ILGFW.....VVG.....K.....EK.....M..G.....E...D..... : 111
01PTHDECJN : .K.RGIQKNCPL.---.G...IIFWIMI..KT-.D.....RD.E.....IR.E.....I..N.....G..... : 110
97PTINSA43 : .K..GIWKNC...---.....FIWL...G..A.PR.....DT.....I..E.....N.A..... : 111

```

V1

V2 (CH01, PG9)

N160K

N7 glycan (197)

```

      120      *      140      *      160      *      180      *      200      *      220      *
HXB2      : DQSLKPCVKLTPLCVSLKCTDL--KNDTNTNSSSGRMIMEKGEIKNCSEFNISTSIRGKVQKEYAFFYKLDIIPID-----NDTTSYKLTSCNTSVITQACPKVSFEPIPIHYC : 218
JR FL      : .....T.N.K.V---.A...TNG.EGT-.R.....T....DE.....L....VV.....NN...R.I..D.....I..... : 215
93A0HDC250 : .E.....I....T.N...ARLQTNNS.T...PE.-----T.E..D.RK...L..RQ.V.Q.NNGIDKGTSNYSD.V.IN....K.....Q..... : 219
08A034HDP  : .....T.N.NSIS-N.I.DS.DTVTSN--G.D.M.....VT.ELKD.KK...L..R...VSLNKNSS--ENSSE.R.IN..S.TV.....N.D..... : 220
01PTHDECJN : .....Q.....T.D.H.YS-.E..NTGMGED.---Y..T.EL.D.K.V.SL..RP.VVKLNEA-----NSST.R.IN...A.....T..... : 216
97PTINSA43 : .....T.N...FG-NTT.KNT..NWET-....M.....T....D.M....L....VV..EENKNSSGNYSN.RMI.....T..... : 224

```

CD4bs (Loop D) N280D V3 loop GPGR/Q motif N332

240 \* 260 \* 280 \* 300 \* 320 \* 340

HXB2 : APAGFAILKCNNKTFNGTGPCTNVSTVQCTHGIRPVVSTQLLNGSLAEEEVIRSVNFTD<sup>N</sup>AKTIIVQLNTSVEINCTRPNNNTRKRIRIQ<sup>R</sup>GPGR<sup>A</sup>AF-VTIGKIGNMRQAHC<sup>N</sup> : 332

JR FL : .....D.....K...K.....D...N.....KE.....S.H.--.....YT.GEI..DI..... : 328

93A0HDC250 : .....D.K.....S.....K.....I...II...K.ISN.....HT...E.I.LA.....S...--...QVLFA.GEI..DI...Y.. : 332

08AO34HDP : ...Y.....H.....K.....I...TKDLNVTQN...H.KEH...L.....SV...--...Q..YA.NAI..DI..... : 333

01PTHDECJN : .....D.G.S.A.T.R.I.....K..A.....GI...E.I.N.....KEP.K.....RG.H.--...T.YAAGEI...I..... : 329

97PTINSA43 : .....DNK.....I...E.....T...H..E.I.....S...KRS.S.--.....WT.GEVT.DI..... : 337

CD4bs (VRC01-CD4 binding loop) V4 region CD4bs (VRC01-beta20/21)

\* 360 \* 380 \* 400 \* 420 \* 440 \* 460

HXB2 : ISRAKWNNTLKQIASKLREQFGNNKTIIFKQSSGGDPEIVTHSFNCGGEFFYCNSTQLFNSTW-FNST-----WSTEGSNNTEGS-----DTITLPCRIKQIINMWQKV<sup>G</sup>GKAMY : 435

JR FL : .....D.....VI.....E-....V.NH.....M.....-N.-----N.....-N.....E..... : 426

93A0HDC250 : .N.TS..S..HKVIG..K.H.K-....S.EPA....Q....T.....TSE...LSRL..GSGEETSNK.H-----VR...R..Q.I. : 430

08AO34HDP : .TEG...K..YEV.K..K.Y.P-.R..K.NS.....L.....A.....TSL..E---R.V.ESNVTN.NS-T.S.TANNASISDKN.....L.....A.R... : 442

01PTHDECJN : V.KTD..R..Q.V.IQ.K.H.T.ATR...NK.....L..T.....TSN....S.-E...M--TSNI.SE.-----Q.....R..Q... : 430

97PTINSA43 : ..G.Q.....IH.VK..K.KL.-....V.N.....M.....R.....P....L--LSN.--W.S.K.I-----N.....L..E..... : 442

G458Y CD4bs (VRC01- beta23/24) V5

end of gp120t

gp120/gp41 interface (PGT151)

```

      *      480      *      500      *      520      *      540      *      560      *
HXB2   : APPISGQIRCSSNITGLLLTRDGCNSN--NESEIFRPGGGDMRDNRSELYKYKVVKIEPLGVAPTAKARRVYQREKRAVGIGALFLGFLGAAGSTMGAASMTLTVQARQLLSGI : 548
JR FL   : ....R.....INE--GT.....K.....V.....L..... : 539
93A0HDC250 : ...A.N.T.T.....P.--GTN.T..T.....E..V.I.....E.....M..V.....I....V..... : 543
08A034HDP : ...E.N.I.R.....V...TVN.---T.T..I...K.....E.K...I..G.....E.....V.....L..A...V.... : 553
01PTHDECJN : ...P.V...E.....T.--G..T..E.....R.....E.....V.....I..... : 542
97PTINSA43 : ...R...N...K...I.....NS.GT.T.....N.K.....L...I...Q.....A.L--M.....V.....L..... : 555

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N611A gp120/gp41 interface (PGT151)

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      580      *      600      *      620      *      640      *      660      *      680      *
HXB2   : VQQQNLLRAIEAQQHLLQLTVWGIKQLQARILAVERYIKDQQLGIWGCSCGLICTTAVPWNASWSNKSLEQIWNHTTWMEWDREINNYTSLIHSLEESQNQQEKNEQEELLEL : 663
JR FL   : .....RM.....V.....G.....DR...NM....E...D...E.YT..... : 654
93A0HDC250 : ...S...K.....K.....V.....T...S.....Y.E..DNM..L..EK.....GI.YN...A..T.....D..A : 658
08A034HDP : ...S.....T.VV.I.....S.....Q.D...NM...Q.....S...YT.YQ.L...HI.....KD..A : 668
01PTHDECJN : ...S.....K.....V..L.A.....T...S.....TYDS..GNM..LQ..K..S...YT.YD...K.....D..A : 657
97PTINSA43 : ...K...N.....V.....N...D.....DT..HNM....E...D...D..YT.L.K..... : 670

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W672A MPER (4E10/2F5)

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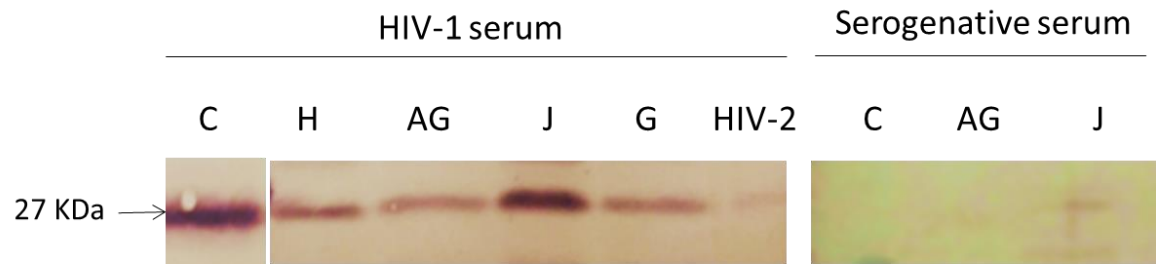
          700          *          720          *          740          *          760          *          780          *          800
HXB2      : DKWASLWNWFNITNWLWYIKLFIMIVGGLVGLRIVFAVLSIVNRVRQGYSPLSFQTHLPTPRGPDREGEIEEGGERDRDRSIRLVNGLSLALIWDLRLSLCLFSYHRLRDLILLIV : 778
JR FL     : .....D..K.....I.....I.....T.....L..A.....G....F....V.....T. : 769
93A0HDC250 : .....D.S.....I.....I.....I.....A.....L..LI.A.TEV...G....G...QG.T.....S.F...A.....FI..A : 773
08A034HDP : .S.N...S...S.....I.....I.....I.....A.I.....LT.N....LGR.....Q....V...S.F.P.V.....C.RQ...CI... : 783
01PTHDECJN : .....D..S.....RI..I.....I.....T.I.....LTHHQ.E....R...G...Q....V...S.F.T.V.....FA..A : 772
97PTINSA43 : .....D.SK.....I.....I.....A.....RF.VR.....Q.....AG.F.EI..V.....RL..... : 785

          *          820          *          840          *          860          *          880          *
HXB2      : TRIVELLGR-----GWEALKYWNLLQYWSQELKNSAVSLNATAIAVAEGTDRVIEVVQGACRAIRHIPRRIRQGLERILL : 856
JR FL     : .....-----...V.....I..AL.RTY...L...T.....A.. : 847
93A0HDC250 : A.T..T....-----...I...LG..AV..R.....I...T...V.....I..IA.RVF...I.....F..A.. : 851
08A034HDP : A.A.....SSLRGLQR.....LKS.V...GL...K..IN..D.I.....I...I.R.....N...T.L..F.AP.. : 868
01PTHDECJN : V.T.....HSSLKGLRL.....LK...S..G.....IN..DT.....NW...I..IG.RTG...CN.....S.. : 857
97PTINSA43 : ..T..F....-----.....L.I.L.R.F...L...T.....A.. : 863

```

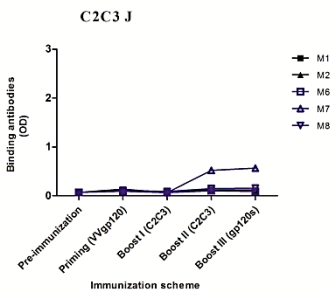
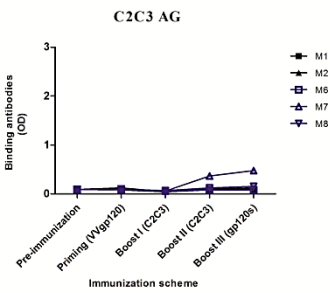
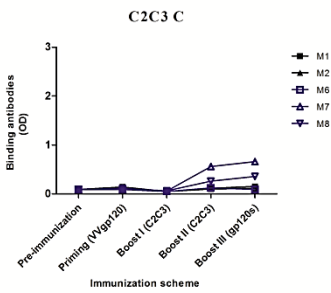
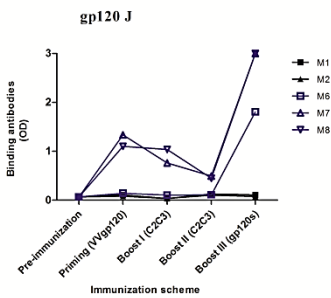
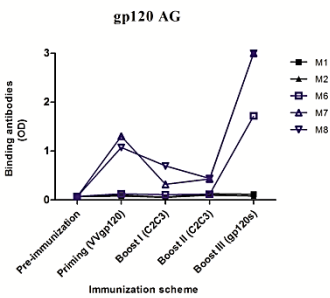
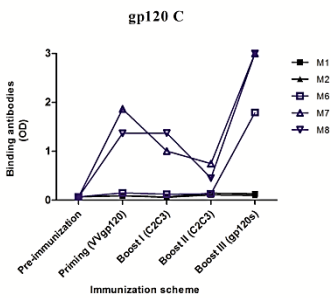
**Figure S2- Amino acid sequence alignment of envelope sequences included in the present study and location of neutralizing epitopes.**

Amino acid sequences of the envelope glycoproteins of isolates 93A0HDC250 (clade J), 08A034HDP (clade C), 01PTHDECJN (clade CRF02\_AG) and 97PTINSA43 (clade B) used to produce the immunogens of the study. The location of the neutralizing epitopes is indicated in relation to the reference HIV-1 strains HXB2 (KO3455) and JR-FL (AY669728) also included in the alignment. Location and name of the neutralizing epitopes and corresponding NAb are shown in red. Amino acids corresponding to an epitope are shown in bold letters. Specific mutations that disrupt the neutralizing epitopes are shown in yellow. N7 glycan (position 197 in HXB2) is shown in purple. GPGR/Q motif is shown in light blue.

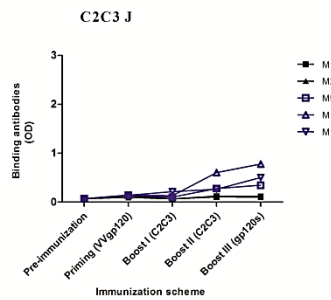
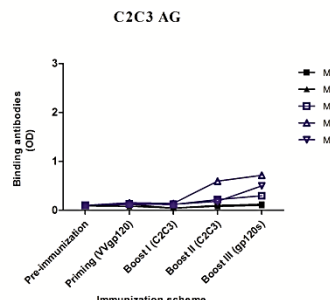
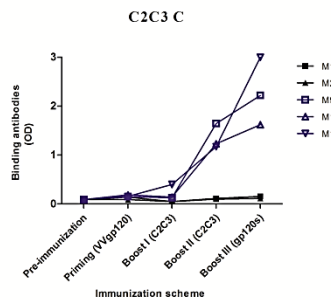
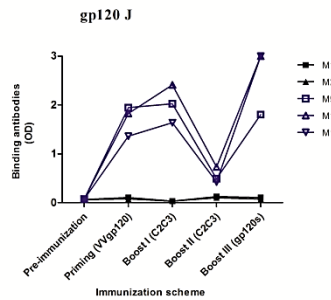
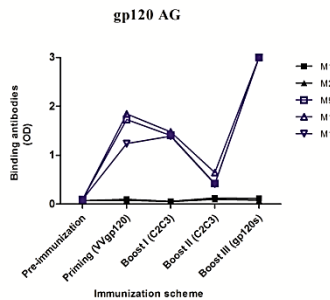
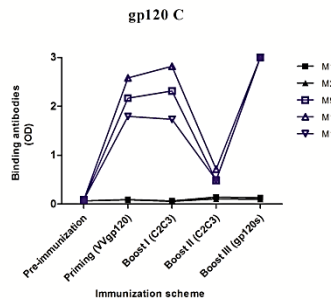


**Figure S3** - Western-Blot analysis of the antigenic reactivity of C2V3C3 polypeptides. C2V3C3 polypeptides from HIV-1 clades C, H, CRF02\_AG, J and G were expressed in *Escherichia coli* strain TOP10 and purified as indicated in Material and Methods. Recombinant polypeptides were incubated with sera from HIV-1 infected individuals (A) and with sera from healthy individuals (B). HIV-2 C2V3C3 polypeptide was used as positive control for this experiment because it has about the same size of HIV-1 C2V3C3 and it shows some cross-reactivity with HIV-1 antiserum.

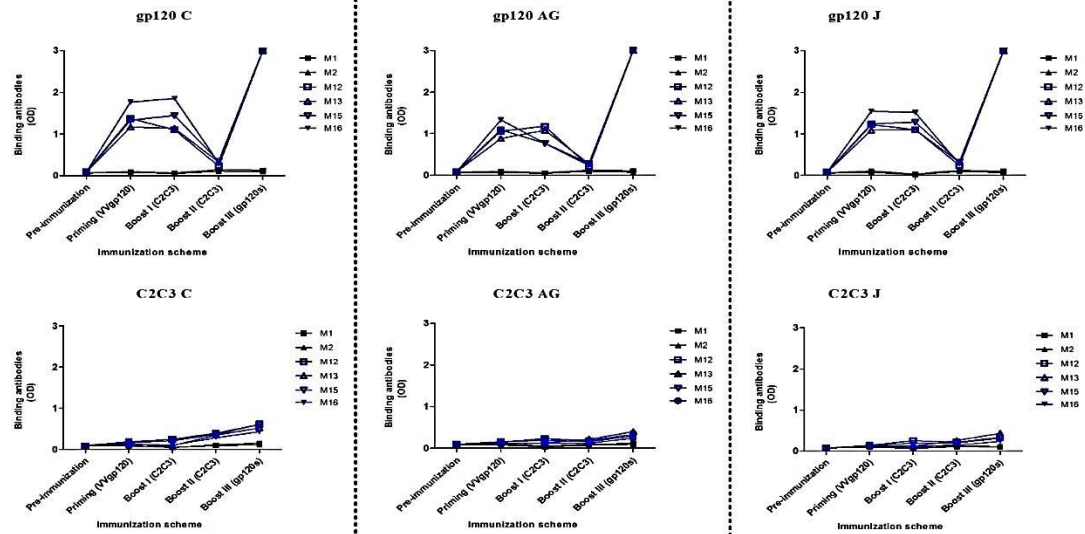
Group 3



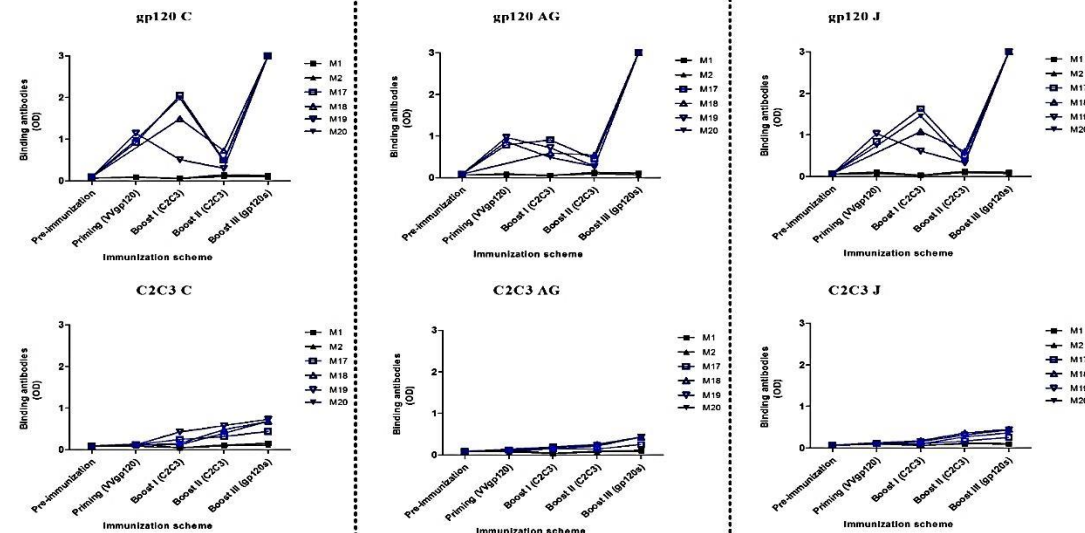
Group 4



## Group 5

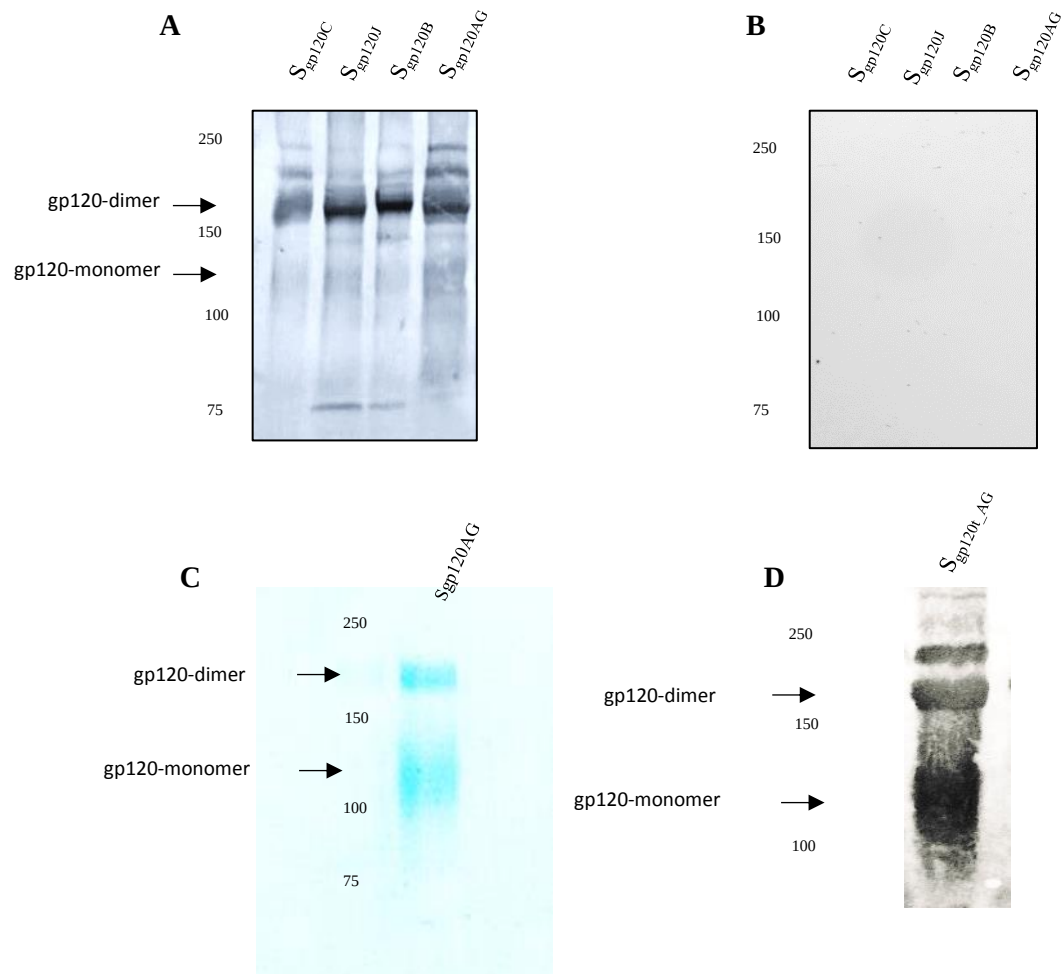


## Group 6



**Figure S4 – Evolution of the binding antibody responses against Sgp120t glycoproteins and C2V3C3 polypeptides in BALB/c mice in the pilot study.** Mice from groups 3 and 4 were primed with VV expressing gp120 and boosted with C2V3C3 polypeptides and Sgp120t from clade C. Mice from group 5 were primed VV expressing gp120 and boosted with C2V3C3 polypeptides and Sgp120t-AG; Mice from group 6 were primed with VV expressing gp120 and boosted with C2V3C3 polypeptides and Sgp120t-J. For all mice, the schedule of immunization included one priming and three boosts at days 15, 30 and 45. Fifteen days after each immunization, sera were collected and assayed for the presence of binding antibodies against HIV-1 immunogens. Blue lines represent immunized mice from the respective group; black lines represent mice from control group (G1-M1-M2).





**Figure S5- Western blot analysis of the reactivity of rabbits' serum with Sgp120t glycoproteins from different clades.** A) Serum reactivity with Sgp120t from clades B, C, J and AG; B) Reactivity with serum from non-immunized rabbit; C) BlueSafe protein stain (Nzytech, Portugal) of gp120t-AG purified by lectin affinity chromatography analysed by 7.5% SDS-PAGE; D) Western-blot analysis of lectin affinity chromatography purified gp120t-AG using sera from immunized rabbit. Precision Plus Protein All Blue Standards (10-250 kDa) was used as a molecular weight marker.

**Table S1-** Characterization of the nine HIV-1 primary isolates included in the study

| Sample     | Year | Origin   | Co-receptor use | Env subtype |
|------------|------|----------|-----------------|-------------|
| 93AOHDC249 | 1993 | Angola   | CCR5            | U           |
| 93AOHDC250 | 1993 | Angola   | CCR5            | J           |
| 93AOHDC251 | 1993 | Angola   | CCR5            | H           |
| 93AOHDC252 | 1993 | Angola   | CCR5            | U           |
| 93AOHDC253 | 1993 | Angola   | CCR5            | J           |
| 08AO34HDP  | 2008 | Angola   | CCR5            | C           |
| 01PTHDECJN | 2001 | Angola   | CCR5            | CRF02_AG    |
| 97PTINSA43 | 1997 | Portugal | CCR5            | B           |
| 00PTEBB    | 2000 | Portugal | CCR5            | G           |

**Table S2-** Primers used for polymerase chain reaction amplification of HIV-1 *Env*, C2V3C3 and truncated gp120.

| Amplified fragment | Name                       | Orientation | Sequence (5' - 3') <sup>1</sup>                        | Clade          | HXB2 position |
|--------------------|----------------------------|-------------|--------------------------------------------------------|----------------|---------------|
| Full-length Env    | PBENV1                     | Forward     | CTATGGCAGGAAGAA<br>GCGG                                | All            | 5968-5986     |
|                    | PBENV2                     | Forward     | CCACTGTCTTCTGCTCTT<br>TC                               | All            | 6203-6223     |
|                    | PBENV3                     | Reverse     | AGTCATTGGTCTTARAG<br>GTAC                              | All            | 9036-9016     |
|                    | PBENV4                     | Reverse     | TTTGACCACTTGCCHC<br>CCAT                               | All            | 8797A881<br>7 |
| Truncated gp120    | RC1gp120 <i>SalI</i>       | Forward     | TTGTGT <u>GTCGAC</u> GAA<br>AGAGCAGAAGAYAGT<br>GGC     | All            | 6203-6223     |
|                    | RC2gp120BCS<br><i>all</i>  | Reverse     | TTCTGT <u>GTCGAC</u> CTAA<br>TATTTATATAATTCAC<br>TTCTC | B,C            | 7661-7682     |
|                    | RC2gp120GA<br><i>GSalI</i> | Reverse     | TTCTGT <u>GTCGAC</u> CTAA<br>TACTTATATAATTCAC<br>TTCTC | G,CRF0<br>2_AG | 7661-7682     |
|                    | RC2gp120J <i>SalI</i>      | Reverse     | TTCTGT <u>GTCGAC</u> CTAA<br>TATTTATATAACTCAC<br>TCCTC | J              | 7661-7682     |
|                    | RC2gp120H <i>SalI</i>      | Reverse     | TTCTGT <u>GTCGAC</u> CTAA<br>TATTTATATAGCTCAC<br>TTCTC | H              | 7661-7682     |
| C2-V3-C3           | HIV1EPIT11                 | Forward     | TGTGGATCCCCAATTC<br>CYATACATTATTG                      | All            | 6858-6878     |
|                    | HIV1EPIT12                 | Reverse     | TGAAAGCTTTCATCA<br>GAAAAATTCYCCTCY<br>AC               | All            | 7374-7392     |

<sup>1</sup> Underlined letters indicate the restriction site for *SalI*; the stop codon **CTA** is indicated in bold letters.

**Table S3-** Primers used for sequencing of HIV-1 *env* gene segments

| Primer  | Orientation | Sequence (5' - 3')   | HXB2 position |
|---------|-------------|----------------------|---------------|
| PBSEQ1  | Forward     | AGCYTAAAGCCATGTGT    | 6567 – 6583   |
| PBSEQ2  | Reverse     | ACACATGGCTTTARGCT    | 6567 – 6583   |
| PBSEQ3  | Forward     | CAGTACAATGTACACA     | 6955 – 6970   |
| PBSEQ4  | Reverse     | TGTGTACATTGTACTG     | 6955 – 6970   |
| PBSEQ5  | Forward     | CATAGTTTTAATTGTRGAGG | 7344 – 7363   |
| PBSEQ6  | Reverse     | CCTCYACAATTAAAACTATG | 7344 – 7363   |
| PBSEQ13 | Forward     | GGACAATTGGAGAAGTGAA  | 7652 – 7670   |
| PBSEQ7  | Forward     | GAGAGAAAAAAGAGCAGT   | 7745 – 7762   |
| PBSEQ8  | Reverse     | ACTGCTCTTTTTTCTCTC   | 7745 – 7762   |
| PBSEQ9  | Forward     | ATCTGCACCACTAATGT    | 8031 – 8047   |
| PBSEQ10 | Reverse     | ACATTAGTGGTGCAGAT    | 8031 – 8047   |
| PBSEQ11 | Forward     | CCTGTGCCTCTTCAGCTACC | 8510 – 8529   |
| PBSEQ12 | Reverse     | GGTAGCTGAAGAGGCACAGG | 8510 – 8529   |

**Table S4-** Characteristics of the global panel of twelve tier 2 HIV-1 Env-pseudoviruses used in neutralization assays.

| Env- pseudoviruses | HIV-1 Clade | Origin   | Year |
|--------------------|-------------|----------|------|
| TRO11              | B           | Italy    | 1995 |
| 25710              | C           | India    | 1999 |
| 398F1              | A           | Tanzania | 2001 |
| X2278              | B           | Spain    | 2007 |
| BJOX2000           | CRF07_BC    | China    | 2007 |
| X1632              | G           | Spain    | 2004 |
| CE1176             | C           | Malawi   | 2004 |
| 246F3              | AC recomb   | Tanzania | 2001 |
| CH119              | CRF07_BC    | China    | 2004 |
| CE0217             | C           | Malawi   | 2007 |
| CNE55              | CRF01_AE    | China    | 2007 |
| CNE8               | CRF01_AE    | China    | 2006 |

**Table S5-** Baseline data of the neutralization assays performed in mice and rabbits

| Viruses                                      | Strains<br>(pseudoviruses and<br>primary isolates) <sup>1</sup> | Relative luminescence units [mean, of 3-5 measurements<br>(standard deviation)] |                            |                              |                 |                            |                              |
|----------------------------------------------|-----------------------------------------------------------------|---------------------------------------------------------------------------------|----------------------------|------------------------------|-----------------|----------------------------|------------------------------|
|                                              |                                                                 | Mice (main study)                                                               |                            |                              | Rabbits         |                            |                              |
|                                              |                                                                 | Virus only                                                                      | Virus + Pre<br>immune sera | % Baseline<br>Neutralization | Virus only      | Virus + Pre<br>immune sera | % Baseline<br>Neutralization |
| <b>HIV-1<br/>(Tier 1<br/>neutralization)</b> | NL4.3                                                           | nd                                                                              | nd                         | -                            | 49,772 (1,941)  | 61,596 (1,897)             | -23                          |
|                                              | SG3.1                                                           | 43,523 (1,700)                                                                  | 68,037 (3,164)             | -56                          | 52,873 (3,712)  | 76,596 (7,270)             | -45                          |
| <b>HIV-1<br/>(Tier 2<br/>neutralization)</b> | PCNE8                                                           | nd                                                                              | nd                         | -                            | 19,858 (673)    | 16,953 (719)               | 15                           |
|                                              | PX2278                                                          | 86,303 (4,569)                                                                  | 57,898 (1,630)             | 33                           | 48,525 (4,538)  | 42,368 (1,737)             | 13                           |
|                                              | TRO11                                                           | 42,267 (1,827)                                                                  | 53,496 (4,414)             | -27                          | 89,112 (10,972) | 107,804 (23,459)           | -21                          |
|                                              | PCH119                                                          | 105,465 (3,693)                                                                 | 141,512 (9,788)            | -34                          | 108,874 (1,981) | 130,004 (30,612)           | -19                          |
|                                              | CNE55                                                           | 39,115 (2,552)                                                                  | 68,500 (6,939)             | -75                          | 34,951 (1,876)  | 45,604 (2,688)             | -30                          |
|                                              | 398F1                                                           | 22,453 (2,089)                                                                  | 18,073 (2,185)             | 20                           | 43,156 (3,361)  | 37,602 (821)               | 13                           |
|                                              | X1632                                                           | 48,372 (3,504)                                                                  | 88,902 (7,736)             | -84                          | 34,740 (3,778)  | 46,808 (2,275)             | -35                          |
|                                              | PCE1176                                                         | 17,543 (1,253)                                                                  | 12,943 (932)               | 26                           | 59,491 (3,091)  | 71,267 (2,091)             | -20                          |
|                                              | 25710243                                                        | 46,636 (4,047)                                                                  | 46,355 (2,068)             | 1                            | 125,840 (6,453) | 158,903 (24,945)           | -26                          |
|                                              | PCE0217                                                         | 71,450 (7,997)                                                                  | 146,026 (10,781)           | -104                         | 104,005 (7,239) | 120,531 (674)              | -16                          |
|                                              | BJOX2000                                                        | 27,445 (3,300)                                                                  | 39,308 (5,198)             | -43                          | 73,659 (11,352) | 113,412 (21,529)           | -54                          |
|                                              | 246F3                                                           | 25,363 (3,224)                                                                  | 46,895 (1,863)             | -85                          | 49,245 (9,551)  | 68,287 (7,031)             | -39                          |
|                                              | 93HDC253*                                                       | 19,141 (5,025)                                                                  | 22,644 (2,598)             | -18                          | 20,637 (416)    | 19591 (187)                | 5                            |
|                                              | 01PTCJN*                                                        | 32,035 (2,863)                                                                  | 84,374 (3,581)             | -163                         | 52,518 (4,809)  | 49,207 (6,754)             | 6                            |
|                                              | 93HDC250*                                                       | 427,353 (45,718)                                                                | 487,798 (26,556)           | -14                          | nd              | nd                         | -                            |
|                                              | 93AOHDC249*                                                     | nd                                                                              | nd                         | -                            | 63,077 (12,006) | 65,420 (756)               | -4                           |
|                                              | 93AOHDC252*                                                     | nd                                                                              | nd                         | -                            | 43,723 (2,192)  | 41,160 (2,464)             | 6                            |
| <b>HIV-2</b>                                 | HCC19.03*                                                       | nd                                                                              | nd                         | -                            | 46,119 (4,595)  | 43,684 (2,468)             | 5                            |
|                                              | AUC*                                                            | nd                                                                              | nd                         | -                            | 44,255 (3,737)  | 40,973 (2,683)             | 9                            |

|            |   |                |                |    |                |              |   |
|------------|---|----------------|----------------|----|----------------|--------------|---|
| <b>VSV</b> | - | 77,943 (8,718) | 68,468 (8,056) | 12 | 28,651 (2,453) | 28,170 (440) | 2 |
|------------|---|----------------|----------------|----|----------------|--------------|---|

<sup>1</sup>Primary isolates are indicated with an asterisk; nd- not one.