CD4 downregulation precedes Env expression and protects HIV-1-infected cells from ADCC mediated by non-neutralizing antibodies

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SUMMARY

HIV-1 envelope glycoprotein (Env) conformation substantially impacts antibody-dependent cellular cytotoxicity (ADCC). Envs from primary HIV-1 isolates adopt a prefusion “closed” conformation, which is targeted by broadly-neutralizing antibodies (bnAbs). CD4 binding drives Env into more “open” conformations, which are recognized by non-neutralizing Abs (nnAbs). To better understand Env-Ab and Env-CD4 interaction in CD4+ T cells infected with HIV-1, we simultaneously measured antibody binding and HIV-1 mRNA expression using multiparametric flow cytometry and RNA-flow fluorescent *in situ* hybridization (FISH) techniques. We observed that *env* mRNA is almost exclusively expressed by HIV-1 productively-infected cells that already downmodulated CD4. This suggest that CD4 downmodulation precedes *env* mRNA expression. Consequently, productively-infected cells express “closed” Envs on their surface, which renders them resistant to nnAbs. Cells recognized by nnAbs were all *env* mRNA negative, indicating Ab binding through shed gp120 or virions attached to their surface. Consistent with these findings, treatment of HIV-1 infected humanized mice with the ADCC mediating nnAb A32 failed to lower viral replication or reduce the size of the viral reservoir. These findings confirm the resistance of productively-infected CD4+ T cells to nnAbs-mediated ADCC and question the rationale of immunotherapy approaches using this strategy.

60 **INTRODUCTION**

61 Highly active antiretroviral therapy (ART) efficiently suppresses HIV-1 replication and
62 significantly increase the life expectancy of people living with HIV-1 (PLWH) (1, 2).
63 However, it has become evident that even lifelong ART cannot eradicate the virus. Viral
64 reactivation and rebound can occur upon treatment interruption due to the presence of a
65 latent reservoir, persisting mainly in long-lived memory CD4+ T cells (3-6). New
66 approaches aimed at eradicating or functionally curing HIV-1 infection by targeting and
67 eliminating productively or latently-infected cells are needed. Monoclonal antibodies
68 (mAbs) are attractive therapeutics for HIV cure strategies, since they target virus specific
69 antigens and have the potential to harness host immune responses such as antibody-
70 dependent cellular cytotoxicity (ADCC). The HIV-1 envelope glycoprotein (Env) is the only
71 viral antigen that is present on the surface of HIV-1-infected cells, thus representing the
72 main target for immunotherapy-based strategies (7). In recent year, several clinical trials
73 explored broadly-neutralizing antibodies (bnAbs) as therapeutic agents to reduce the HIV-
74 1 reservoir via Fc-mediated effector functions (8). Monotherapy or combination of bnAbs
75 targeting multiple regions of the Env trimer including the V3 glycan supersite (10-1074,
76 PGT121), the V2 apex (PGDM 1400, CAP256-VRC26), or the CD4 binding site (CD4bs)
77 (3BNC117, N6, VRC01, VRC07-523) are currently under investigation. Administration of
78 bnAbs to humans has been found to be safe and effective in lowering viremia and
79 maintaining viral suppression for varying periods of time after treatment interruption (9-
80 15). However, recent data suggest that bnAbs may not be as broad and/or effective as
81 predicted, in part because of circulating viruses with pre-existing resistance to the
82 administered bnAbs (8). So-called “non-neutralizing” Abs (nnAbs) have been evaluated
83 as a potential alternative, because they target highly conserved Env epitopes that are
84 usually occluded in the closed trimer, including the coreceptor-binding site (CoRBS) (16-
85 18), the inner domain of gp120 (19-21) or the gp41 immunodominant domain (gp41ID)

86 (22). These nnAbs are naturally elicited during HIV-1 infection and can mediate potent Fc
87 effector functions (22-28).
88
89 HIV-1 Env is a conformationally flexible molecule that transitions from an
90 unliganded “closed” State 1 conformation to an “open” CD4-bound State 3 conformation
91 (29-31) upon CD4 interaction. Envs from most primary HIV-1 isolates adopt a “closed”
92 conformation that is efficiently targeted by bnAbs, but is resistant to nnAbs (29, 32-36),
93 which target epitopes that are occluded within the unliganded “closed” trimer. Env
94 interaction with CD4 or small CD4-mimetic compounds are known to induce more “open”
95 conformations, thus sensitizing infected cells to ADCC mediated by CD4-induced (CD4i)
96 nnAbs (23, 24, 26, 27, 33, 34, 36-42). To avoid exposing these nnAb epitopes, the Env
97 trimer is stabilized by multiple intermolecular interactions, including between the V1, V2
98 and V3 variable loops as well as the gp120 β20–β21 element, which maintain a “closed”
99 conformation (31, 43, 44). In addition, HIV-1 expresses the accessory proteins Nef and
100 Vpu, which indirectly modulate Env conformation by downregulating CD4 from the surface
101 of infected cells, thus preventing a premature Env-CD4 interaction that would otherwise
102 result in the exposure of CD4i Env epitopes (23, 26). Vpu-mediated counteraction of the
103 restriction factor BST-2 (also named “tetherin”), known to tether viral particles at the
104 surface of infected cells, also reduces cell-surface levels of Env (23, 26, 45, 46).
105
106 Despite advances in understanding Env-Ab and Env-CD4 interactions, the
107 capacity of nnAbs to target and eliminate productively-infected cells by ADCC remain
108 controversial. Several studies reported that cells infected with primary viruses expressing
109 functional Vpu and Nef proteins are resistant to ADCC mediated by CD4i nnAbs, because
110 they maintain surface expressed Env in a “closed” conformation (23, 24, 27, 32-42, 47,
111 48). Other studies report that CD4i nnAbs can mediate ADCC responses, but they have

112 used infectious molecular clones (IMCs) that are defective for Nef expression (49-60).
113 These IMCs contain reporter genes (e.g., the Renilla luciferase [LucR] gene) upstream of
114 the *nef* gene, which significantly reduces Nef expression and thus CD4 downregulation
115 from the surface of infected CD4+ target cells. The resulting premature Env-CD4
116 interaction promotes the artificial exposure of otherwise occluded CD4i epitopes (39, 61,
117 62).
118
119 Here, we used multiparametric flow cytometry and RNA-flow fluorescent *in situ*
120 hybridization (FISH) techniques to characterize cell populations targeted by bNAbs and
121 nnAbs in the context of primary CD4+ T cell infection. We show that *env* mRNA is
122 specifically detected among cells that already downmodulated cell-surface CD4, which
123 renders these cells refractory to recognition by nnAbs. Although some CD4+ cells are
124 bound by nnAbs, they are all negative for *env* mRNA, indicating that they are not
125 productively-infected and nnAbs binding is mediated through the recognition of shed
126 gp120 and/or viral particles coated at their cell surface. The same results were obtained
127 with *ex vivo*-expanded CD4+ T cells isolated from PLWH. Finally, the ADCC mediating
128 nnAb A32 failed to reduce HIV-1 replication and the size of the viral reservoir in a
129 humanized mouse model (hu-mice) that supports Fc-effector functions. These findings
130 raise questions about curative immunotherapy-based strategies that rely solely on nnAbs,
131 specifically those targeting CD4i epitopes.

132 **RESULTS**

133

134 **Expression of CD4, p24 and Nef defines recognition of HIV-1 infected CD4+ T cells**
135 **by nnAbs and bnAbs**

136 To characterize cell populations targeted by nnAbs and bnAbs in HIV-1-infected
137 primary CD4+ T cells, we used multiparametric flow cytometry to simultaneously probe
138 CD4 and viral proteins expression. Activated primary CD4+ T cells were mock-infected or
139 infected with the transmitted-founder virus CH077 (CH077TF). Two days post-infection,
140 cells were stained with a panel of nnAbs and bnAbs, followed by appropriate secondary
141 Abs. Cells were then stained for cell-surface CD4 prior to staining for intracellular HIV-1
142 p24. Given that the *nef* gene is abundantly expressed during the early phase of the HIV-
143 1 replication cycle (63, 64), we also evaluated the expression of this accessory protein by
144 intracellular staining as previously described (61). The different cell populations were
145 gated based on cell-surface CD4 and intracellular p24 detection as shown in Figure 1A.
146 As expected, uninfected $CD4^{+}p24^{-}$ cells were not recognized by bnAbs of various
147 specificities (PGT126, PG9, 3BNC117, PGT151 and 2G12) (Figure 1B-D). Interestingly,
148 this population was efficiently recognized by CD4i nnAbs targeting the coreceptor-binding
149 site (17b) or the gp120 inner domain (A32, C11) as well as a pool of purified
150 immunoglobulins from PLWH (HIV-IG). However, the $CD4^{+}p24^{-}$ cells were not recognized
151 by the CD4i gp41-specific nnAbs F240. The absence of binding by bnAbs and F240
152 suggested that these cells were likely coated with shed gp120 rather than presenting CD4-
153 bound cell-surface Env trimer. This is in line with previous work showing that uninfected
154 CD4+ T cells expose CD4i epitopes on their cell surface after interacting with gp120 shed
155 from productively-infected cells within the same culture (33, 65, 66). Indeed, introduction
156 of a CD4bs (D368R) mutation into CH077TF that prevents Env-CD4 interaction (17, 23,

157 67), abrogated the recognition of the $CD4^{+}p24^{-}$ population by all gp120-specific nnAbs
158 tested (Figure S1A-B).
159
160 In addition to productively infected CD4+ T cells, which efficiently downregulated
161 CD4 ($CD4^{low}p24^{high}$), we identified a subset of $CD4^{+}p24^{low}$ cells, as previously reported (51,
162 58, 68-72). This $CD4^{+}p24^{low}$ subset was efficiently recognized both by the gp120-specific
163 nnAbs and HIV-IG, but was resistant to bnAbs binding. This population was also
164 recognized by the gp41-specific F240 nnAb, suggesting the presence of trimeric Env
165 bound to CD4. However, recognition of the $CD4^{+}p24^{low}$ cells by CD4i nnAbs was
166 substantially reduced upon the introduction of the D368R mutation (Figure S1A-B). This
167 mutation also specifically reduced the proportion of $CD4^{+}p24^{low}$ cells (Figure S1C). These
168 findings, along with the observation that these cells do not express Nef (Figure 2) suggest
169 that $CD4^{+}p24^{low}$ cells are not productively infected, but contain CD4-Env complexes on
170 their surface resulting from the binding of shed gp120 and/or viral particles.
171
172 As expected, cells that expressed high level of p24 ($CD4^{low}p24^{high}$) were poorly
173 recognized by nnAbs. We reasoned that this was because they efficiently downregulated
174 cell-surface CD4, which precludes premature Env triggering, and thus prevents the
175 exposure of normally occluded epitopes (23, 26, 33). $CD4^{low}p24^{high}$ cells also expressed
176 Nef (Figure 2) and were efficiently recognized by bnAbs, known to preferentially recognize
177 Env in its "closed" conformation (Figure 1B-D). However, when we used a *nef*-defective
178 virus these cells were efficiently recognized by nnAbs (Figure 1E-H), in agreement with
179 previous observations (26, 33, 37, 39, 61, 73, 74).
180
181 To test whether our findings extend to IMCs widely used in the ADCC field (28, 35,
182 53, 55, 68, 72, 75-77), we infected primary CD4+ T cells with with viruses produced from

183 a pNL4.3 backbone expressing different Envs, including BaL and NL4.3. As shown in
184 Figure S2A-B, the patterns of bnAbs and nnAbs binding were very similar to those
185 observed for CH077TF. The $CD4^{+}p24^{-}$ and $CD4^{+}p24^{low}$ cell populations were preferentially
186 recognized by the nnAbs, while the $CD4^{low}p24^{high}$ cells were efficiently targeted by the
187 bnAbs. Interestingly, despite similar levels of productive infection ($CD4^{low}p24^{high}$, Figure
188 S2C), an enrichement of the $CD4^{+}p24^{low}$ population was detected in the context of
189 infection with the NL4.3 and BaL Envs as compared to Envs from primary viruses
190 (CH058TF, CH040TF, SF162 and YU2) (Figure S2D). The reasons for this are unclear
191 but may be due to greater gp120 shedding of the tier 1 NL4.3 and BaL Envs.
192
193 Taken together, our results indicate that cells recognized by nnAbs express high
194 levels of CD4, are either $p24^{low}$ or $p24^{-}$, and are negative for Nef expression. In contrast,
195 bnAbs recognize cells that efficiently downregulate CD4 and express high level of p24 and
196 Nef proteins.
197
198 **e*nv* mRNA is predominantly detected in cells that already downregulated CD4**
199 To better understand the underlying mechanisms behind the differential recognition
200 of infected cells by bNAbs versus nnAbs, we used a previously described RNA-flow
201 cytometric fluorescence *in situ* hybridization (RNA Flow-FISH) method (78-80). This
202 method identifies productively-infected cells by detecting cellular HIV-1 mRNA by using *in*
203 *situ* RNA hybridization and intracellular Ab staining for the HIV-1 p24 protein. In the context
204 of these experiments, *env* and *nef* mRNA probes were used to identify productively-
205 infected CD4+ T cells. Briefly, primary CD4 T cells were mock-infected or infected with
206 the CH077TF IMC. Two days post-infection, cells were stained for surface CD4 before
207 fixation and permeabilization to allow detection of the HIV-1 p24 antigen and HIV-1
208 mRNAs. Cell populations were first defined based on their cell-surface CD4 and

209 intracellular p24 co-expression, as presented in Figure 3A. Productively-infected cells
210 were identified as *nef* mRNA+/*env* mRNA+.The vast majority of cells with detectable cell-
211 surface CD4 ($CD4^{+}p24^{-}$ or $CD4^{+}p24^{low}$) were negative for HIV-1 mRNA (Figure 3B-C),
212 while cells that efficiently downmodulated CD4 ($CD4^{low}p24^{low}$ and $CD4^{low}p24^{high}$) were
213 enriched for *nef* and *env* mRNA transcripts (Figure 3B-C; Figure S3). When gating on
214 productively-infected cells based on *nef* and *env* mRNA detection, we confirmed that the
215 $CD4^{low}p24^{high}$ cells represent the major source of productively-infected cells (Figure 3D-
216 E). These results show that *env* mRNA is predominantly expressed by HIV-1-infected cells
217 that already downregulated CD4 ($CD4^{low}p24^{low}$ and $CD4^{low}p24^{high}$) (Figure S3). This
218 analysis also captured stages of infection ($CD4^{low}p24^{low}$) where CD4 is already
219 downmodulated while *env* mRNA expression intensifies, suggesting that CD4
220 downmodulation precedes *env* mRNA expression.
221
222 **Cells targeted by A32 are negative for HIV-1 mRNA**
223 We next combined flow cytometry and RNA flow-FISH methods to compare the
224 capacity of nnAbs and bnAbs to bind productively-infected cells. Mock-infected or
225 CH077TF-infected primary CD4 T cells were first stained with the nnAb A32 or the bNAb
226 PGT126. Cells where then stained for cell-surface CD4 detection, intracellular p24 and
227 HIV-1 mRNAs. Productively-infected cells were identified based on the simultaneous
228 detection of *nef* and *env* mRNA transcripts (Figure 4A). As shown in Figure 4B-C,
229 productively-infected cells (*env*/*nef* mRNA+ cells) were recognized by PGT126, but not by
230 A32. In contrast, *env*/*nef* mRNA- cells were targeted by A32, but not by PGT126. Similar
231 results were obtained when cells were classified by Ab recognition (Figure 4D-F; Figure
232 S4), with the majority of PGT126+ cells co-expressing HIV-1 mRNA. In contrast, cells
233 recognized by A32 were negative for *env* and *nef* mRNA (Figure 4E-F; Figure S4). These

234 results indicate that cells targeted by A32 do not express *env* mRNA (Figure S4), are not
235 productively infected and thus are likely coated with gp120 and/or viral particles.
236
237 ***Ex vivo* expanded CD4+ T cells isolated from PLWH are resistant to ADCC**
238 **responses mediated by nnAbs**
239 Our results indicated that productively infected cells are principally $CD4^{low}p24^{high}$,
240 express *nef* and *env* mRNA and are not recognized by nnAbs. To confirm these findings
241 with primary clinical samples, we expanded CD4+ T cells from PLWH. Briefly, CD4+ T
242 cells were isolated from 6 chronically-infected individuals and activated *ex vivo* using PHA-
243 L/IL-2 (24, 38, 40). CD4+ T cells from people without HIV were used as controls. Viral
244 replication was monitored over time by intracellular p24 staining (Figure 5A). Upon
245 expansion, CD4+ T cells were stained with a panel of bnAbs and nnAb, followed by the
246 appropriate secondary Abs. Cells were stained for cell-surface CD4 prior to the detection
247 of intracellular HIV-1 p24 and Nef proteins. Consistent with the results obtained with IMC
248 infections (Figure 2), productively-infected $CD4^{low}p24^{high}$ cells were the only ones which
249 were also positive for the Nef protein (Figure S5). These cells were efficiently recognized
250 by bnAbs and largely resistant to nnAbs binding (Figure 5C,D, E). In contrast, nnAbs
251 mainly recognized CD4+ cells that were either $p24^{-}$ or $p24^{low}$ as well as negative for Nef
252 expression (Figure 5C, D, E; Figure S5). To evaluate the susceptibility of productively-
253 infected cells to ADCC responses mediated by bnAbs and nnAbs, expanded
254 endogenously-infected cells were used as target cells and autologous peripheral blood
255 mononuclear cells (PBMCs) as effectors using a FACS-based ADCC assay (Figure 6).
256 Consistent with antibody binding, productively-infected $CD4^{low}p24^{high}$ cells were resistant
257 to ADCC mediated by nnAbs, but sensitive to those mediated by bnAbs.
258
259

260 **A32 does not affect HIV-1 replication or the size of HIV-1 reservoir *in vivo*.**

261 Our results indicate that nnAbs, such as A32, target nonproductively-infected
262 CD4+ T cells. It has been suggested that these cells could be in a very early stage of
263 infection, during viral entry, before viral gene expression (81). Specifically, it has
264 previously been shown that non-neutralizing Env epitopes, such as that targeted by A32,
265 become transiently exposed during viral entry and could therefore represent a suitable
266 target for ADCC at this stage (81). We hypothesized that if this was the case, then this cell
267 population should be eliminated by A32 and therefore decrease HIV-1 replication *in vivo*.
268 To evaluate this possibility, we tested whether A32 affected viral replication in humanized
269 mice (hu-mice). Briefly, NOD.Cg-*Prkdc*^{scid} IL2rg$^{-/-}$ Tg(Hu-IL15) (NSG-15) hu-mice
270 engrafted with human peripheral blood lymphocytes (hu-PBL) were infected with the
271 primary isolate HIV-1_{JRCSF} (Figure 7A). This hu-mice model was previously shown to
272 support HIV-1 replication and antibody Fc-effector function *in vivo* (24, 82). Infected hu-
273 mice received nnAb A32 administered subcutaneously (S.C.) at day 6 and 9 post-infection
274 (Figure 7A). As controls, infected mice were also treated with the bnAbs 3BNC117 or its
275 Fc gamma receptor (FcγR) null binding variant (GRLR) (83). Hu-mice were monitored for
276 plasma viral loads (PVLs) and peripheral CD4+ T cells overtime (Figure 7B-C). In the
277 absence of antibody treatment, mice became viremic, reaching an average PVL of $2.2x10^7$
278 copies/mL at day 11. As previously reported (41, 82), viral replication was associated with
279 a loss of peripheral CD4+ T cells. Treatment with 3BNC117 WT significantly reduced viral
280 replication and partially restored CD4+ T cell levels in peripheral blood and several tissues,
281 while A32 failed to do so (Figure 7B,C,D). Interestingly, A32 treatment further reduced
282 CD4+ T cell levels in tissues relative to mock-treated mice (Figure 7D), consistent with its
283 capacity to recognize and eliminate uninfected bystander cells coated with soluble gp120
284 via ADCC (33, 65, 66). This reduction in CD4 T cells count was particularly significant
285 when CD4+ T cell levels where considered across all tissues in mice treated with A32

286 nnAb alone (Figure S6). Finally, treatment with 3BNC117 WT led to a significant reduction
287 of the HIV-1 reservoir in multiple tissues, a phenotype not observed with A32 and not fully
288 achieved by its FcγR null binding variant (3BNC117 GRLR, Figure 7E). These results are
289 in line with previous work demonstrating that bnAbs require Fc effector functions for *in*
290 *vivo* activity (83). These results indicate that A32 does not reduce HIV-1 replication or the
291 size of the reservoir in hu-mice.
292
293
294 **DISCUSSION**
295
296 ADCC represents an effective immune response involved in the clearance of
297 virally-infected cells. This adaptive immune response relies on the capacity of Abs to act
298 as a bridge between infected cells and effector cells. While the Abs recognize infected
299 cells through binding of surface Env via their Fab domain, their Fc domain allows the
300 recruitment and the stimulation of FcγR bearing effector cells, leading to the killing of
301 infected cells. ADCC-mediating Abs therefore represent attractive therapeutics for HIV
302 cure strategies. However, the ability of Abs to recognize productively-infected cells and to
303 mediate ADCC responses depends on Env conformation (73). While bnAbs target
304 epitopes on the prefusion "closed" Env trimer, nnAbs recognize conserved epitopes that
305 are normally occluded but are exposed when Env interacts with CD4 and adopt an "open"
306 conformation (29, 32-36). Here, we provide evidence that *env* mRNA expression is mainly
307 restricted to cells that already downmodulated cell-surface CD4 (Figures 3 and S3). This
308 prevents CD4i Env epitopes exposure on productively-infected cells, thus contributing to
309 their resistance to CD4i nnAbs.
310

311 Non-neutralizing Abs, such as those targeting the CoRBS and the inner domain of
312 gp120 are elicited during natural infection due to exposure to “viral debris” acting as
313 immunodominant decoy (23, 25, 28, 34, 84, 85). It is therefore not surprising that HIV-1
314 has evolved mechanisms to prevent surface Env-CD4 interaction to avoid Env recognition
315 by these nnAbs which have potent Fc-effector functions. HIV-1 utilizes Nef, Vpu and Env
316 to downmodulate CD4 from the surface of infected cells (86). Nef is expressed at high
317 levels early during infection from a multi-spliced transcript (63, 64) and downregulates
318 CD4 by enhancing its internalization and lysosome degradation (87-91). Vpu and Env are
319 expressed from a Rev-depended single spliced bicistronic mRNA later during the viral life
320 cycle (92). Both Vpu and Env interfere with the transport of newly synthesized CD4 to the
321 cell surface (93-95). Using RNA-Flow FISH methods, we show that *env* mRNA is almost
322 exclusively detected in cells positive for *nef* mRNA that efficiently downregulated CD4.
323 The majority of cells expressing HIV-1 mRNA substantially downregulated surface CD4
324 and express high levels of p24 and Nef proteins ($CD4^{low}p24^{high}$ cells). We also captured
325 stage of infection ($CD4^{low}p24^{low}$) where CD4 is already downmodulated while *env* mRNA
326 and p24 are not fully expressed. This suggest that Nef-mediated CD4 downmodulation
327 precedes Env expression (Figure 3 and S3). Accordingly, productively-infected cells failed
328 to expose CD4i epitopes, explaining their resistance to ADCC mediated by nnAbs, while
329 showing susceptibility to ADCC mediated by bNabs (Figure 1, 4, 5 and 6).
330
331 Our findings are consistent with a growing number of studies demonstrating the
332 importance of bnAbs in mediating ADCC against cells productively infected with primary
333 HIV-1 isolates (32, 33, 35). nnAbs recognize productively-infected cells only when CD4 is
334 not properly downregulated, as is the case in infections with Nef defective virus (Figure
335 1). This is also the case when *nef*-deficient IMCs are used for ADCC detection (28, 49-60,
336 69, 71, 76). Most of these IMCs express the Renilla luciferase (LucR) reporter gene

337 upstream of the *nef* sequence and use a T2A ribosome-skipping peptide to promote Nef
338 expression (62). Despite documented evidence of reduced Nef expression (39, 61, 62),
339 these IMCs continue to be used in the ADCC field (51, 52, 54, 55, 57, 58, 96). While some
340 studies have suggested that Vpu can compensate for the absence of Nef (51, 52), our
341 findings refute this. Given its early expression and ability to target cell surface expressed
342 CD4 molecules, Nef plays the most prominent role in CD4 downmodulation (61, 86, 97).
343 In absence of Nef expression, we find that Vpu is not sufficient to downregulate cell-
344 surface CD4, leading to CD4i epitope exposure and efficient nnAbs binding (Figure 1).
345 Other studies reporting Fc-effector functions of nnAbs employed assays unable to
346 differentiate the ADCC responses directed against HIV-1-infected cells versus uninfected
347 bystander cells (45, 49, 76, 98, 99). The presence of uninfected bystander cells coated
348 with gp120 shed from productively-infected cells impacts ADCC measurement by
349 introducing a significant bias toward CD4i nnAbs (33), which is also the case when ADCC
350 assays rely solely on target cells coated with gp120 or inactivated virions (33, 50, 60, 71,
351 72, 100, 101). Utilization of such assays, as well as *nef*-defective IMCs, contribute to the
352 propagation of a misleading concept that nnAbs can effectively mediate ADCC against
353 HIV-1-infected cells. nnAbs such as A32 not only fail to eliminate HIV-1-infected cells, but
354 also have potentially detrimental effects by accelerating the elimination of uninfected
355 bystander cells (33, 66), as shown in tissues of HIV-1-infected humanized mice (Figure 7;
356 Figure S6). In this context, the absence of an antiviral effect of A32 in hu-mice is not
357 surprising. Studies in non-human primate showed that elicitation of A32-like Abs by gp120
358 immunization or passive administration of A32 failed to confer protection against simian-
359 human immunodeficiency virus challenges (77, 102). Similarly, a combination of anti-
360 CoRBS and anti-cluster A (A32) nnAbs proved ineffective in delaying viral rebound after
361 ART interruption in humanized mouse model supporting Fc effector functions (41).
362

363 The inability of A32 to recognize productively-infected cells, to influence viral
364 replication and reduce the size of reservoir in hu-mice is a function of its epitope, which is
365 occluded in the unliganded trimer. To our knowledge, exposure of this epitope at the
366 surface of productively infected cells is possible in only two ways: either by membrane-
367 bound CD4 (26) or the combination of potent small CD4-mimetic compounds (CD4mc)
368 and anti-CoRBS Abs (27, 41). Of note, the cocktail of A32, 17b (CoRBS Ab) and CD4mc
369 was reported to significantly reduce the size of the reservoir in hu-mice (41).
370
371 In conclusion, we show that *env* mRNA is almost exclusively expressed by
372 productively-infected cells that downregulated cell-surface CD4. This suggests that CD4
373 downmodulation precedes Env expression, thus preventing exposure of vulnerable CD4-
374 induced Env epitopes and evading ADCC mediated by nnAbs. These results must be
375 taken into account when considering the use of nnAbs for preventative or cure strategies.
376

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AUTHOR CONTRIBUTIONS

Conceptualization: J.R., G.S, L.Z, P.K., D.E.K and A.F.; **Methodology:** J.R, G.S, J.P., L.Z..,M.D., P.K., D.E.K. and A.F.; **Investigation:** J.R, G.S., L.Z., L.M., M.B., G.B.B., D.C., Y.S., H.K., J.P., H.M., C.B., and G.G.D.; **Resources:** J.R., G.S., J.P., L.Z., L.M., M.B., G.B.B., D.C., F.K., B.H.H., P.K., D.E.K. and A.F.; **Formal Analysis:** J.R., G.S. and L.Z.; **Visualization:** J.R., G.S and L.Z. **Supervision:** M.D., P.K., D.E.K. and A.F.; **Funding acquisition:** J.R. P.K., D.E.K. and A.F.; **Writing – original draft:** J.R., B.H.H., and A.F. **Writing – review & editing:** J.R., G.S., L.Z., J.P., L.M., M.B., G.B.B., D.C., F.K., B.H.H.,Y.S., H.K., H.M., C.B., G.G.D., M.D., P.K., D.E.K. and A.F.

415 **FIGURE LEGENDS**

416

417 **Figure 1. Recognition of HIV-1-infected primary CD4+ T cells by bnAbs and nnAbs.**
418 Primary CD4+ T cells, mock-infected or infected with the transmitted-founder virus CH077,
419 either wild-type (WT) or defective for Nef expression (*nef*-) were stained with a panel of
420 bnAbs and nnAbs, followed with appropriate secondary Abs. Cells were then stained for
421 cell-surface CD4 prior detection of intracellular HIV-1 p24. (A,E) Example of flow cytometry
422 gating strategy based on cell-surface CD4 and intracellular p24 detection. (B,F)
423 Histograms depicting representative staining with bnAbs (Green) and nnAbs (Black)
424 (C,G). Graphs shown represent the median fluorescence intensities (MFI) obtained for at
425 least 6 independent staining with the different mAbs. Error bars indicate means ± standard
426 errors of the means. (D,H) Graphs shown represent the mean MFI obtained with each
427 mAb. Statistical significance was tested using Mann-Whitney U test (** $p<0.01$, ns: non-
428 significant).

429

430 **Figure 2. Nef is expressed in HIV-1-infected cells undergoing CD4 downregulation**
431 **and expressing high levels of p24.**
432 Primary CD4+ T cells, mock-infected or infected with the transmitted-founder virus CH77,
433 either WT or defective for Nef expression (Nef-) were stained for cell-surface CD4 prior
434 detection of intracellular HIV-1 p24 and Nef expression. (A) Histograms depicting
435 representative intracellular Nef staining when gating on $CD4^{+}p24^{-}$, $CD4^{+}p24^{low}$ or
436 $CD4^{low}p24^{high}$ cells. In the context of cells infected with CH77TF Nef-, in the absence of
437 Nef-mediated CD4 downmodulation, the $p24^{high}$ remained $CD4^{high}$ ($CD4^{high}p24^{high}$). (B)
438 Quantification of the median fluorescence intensities (MFI) obtained for 6 independent
439 experiments. Error bars indicate means ± standard errors of the means. Statistical

440 significance was tested using multipe Mann-Withey tests with a Holm-Sidak post-test (**
441 p<0.01, ns: non-significant).
442
443 **Figure 3. HIV-1 late transcripts are mostly detected among cells that downregulated**
444 **CD4.** Purified primary CD4+ T cells, mock-infected or infected with the transmitted-founder
445 virus CH077 WT were stained for cell-surface CD4 prior detection of intracellular HIV-1
446 p24 and *env* mRNA and *nef* mRNA by RNA-flow FISH. (A) Representative example of
447 flow cytometry gating strategy based on cell-surface CD4 and intracellular p24 detection.
448 (B) Representative example of RNA-flow FISH detection of *env* and *nef* mRNA among the
449 different cell populations. (C) Quantification of the percentage of *env* mRNA+ *nef* mRNA+
450 cells detected among the different cell populations in three different donors. (D)
451 Alternatively, productively-infected cells were first identified based on *env* and *nef* mRNA
452 detection (E) Quantification of the percentage of $CD4^{+}p24^{-}$, $CD4^{+}p24^{low}$, $CD4^{low}p24^{low}$ and
453 $CD4^{low}p24^{high}$ cells among the *env* and *nef* mRNA+ cells with three different donors.
454 Statistical significance was tested using one-way ANOVA with a Holm-Sidak post-test
455 (****p<0.0001, ns: non-significant).
456
457 **Figure 4. Productively-infected cells are resistant to recognition by A32**.
458 Purified primary CD4+ T cells, mock-infected or infected with the transmitted-founder virus
459 CH077 WT were stained with A32 or PGT126, followed with appropriate secondary Abs.
460 Cells were then stained for cell-surface CD4 prior detection of intracellular HIV-1 p24 and
461 *Env* mRNA and *nef* mRNA by RNA-flow FISH. (A-C) In a first analysis, HIV-infected cells
462 were identified, then A32 and PGT126 binding was evaluated. (A) Example of RNA-flow
463 FISH gating strategy based on *env* and *nef* mRNA detection. (B) Example of antibody
464 binding among the *env/nef* mRNA- and *env/nef* mRNA+ cell population. (C) Quantification
465 of the percentage of cells recognized by either A32 or PGT126 among the *env/nef* mRNA-

466 and *env/nef* mRNA+ cell population with three different donors. (D-F) In a second
467 alternative analysis, Ab-binding cells were first identied, and the HIV-infection status was
468 then evaluated. (D) Example of flow cytometry gating strategy based on A32 or PGT126
469 binding. (E) Example of *env/nef* mRNA detection among the cells recognized (Ab+) or not
470 (Ab-) by indicated mAbs. (F) Quantification of the percentage of *env/nef* mRNA+ cells
471 among the cells recognized (Ab+) or not (Ab-) by indicated mAbs with three donors.
472 Statistical significance was tested using a two-way ANOVA with a Holm-Sidak post-test (*
473 $p<0.05$,** $p<0.01$, **** $p<0.0001$, ns: non-significant).
474
475 **Figure 5. *Ex vivo* expanded CD4+ T cells isolated from PLWH are preferentially**
476 **targeted by bNAbs.**
477 *Ex vivo* expanded CD4+ T cells from 6 PLWH were stained with bnAbs and nnAbs +/-
478 CD4mc, followed by appropriate secondary Abs. Cells were then stained for surface CD4
479 prior to detection of intracellular HIV-1 p24. (A) Percentage of p24+ upon activation
480 overtime. (B) Example of flow cytometry gating based on CD4 and p24 detection. (C)
481 Histograms depicting representative staining with bnAbs (Green) and nnAbs (Black). (D)
482 Median fluorescence intensities (MFI) obtained with 6 HIV+ individuals. (E) Graphs shown
483 represent the mean MFI obtained with each mAb. Each symbol represent a diffirent HIV+
484 donor. Statistical significance was tested using Mann-Whitney U test (* $p<0.05$, ****
485 $p<0.0001$).
486
487 **Figure 6. *Ex vivo* expanded CD4+ T cells isolated from PLWH are resistant to ADCC**
488 **mediated by nnAbs.**
489 *Ex vivo* expanded CD4 T cells from PLWH were used as target cells, while autologous
490 PBMC were used as effector cells in our FACS-based ADCC assays. (A) Graph shown
491 represent the percentage of ADCC against the $CD4^{low}p24^{high}$ cells with the single mAbs

492 and (B) nnAbs vs bnAbs. (A) Each symbol represent a diffirent HIV+ donor. Statistical
493 significance was tested using unpaired t test (**** p<0.0001).
494
495 **Figure 7. A32 nnAb does not impact viral replication or the size of the reservoir *in***
496 ***vivo***
497 (A) Experimental outline. NSG-15-Hu-PBL mice were infected with HIV-1 JRCSF
498 intraperitoneally. At day 6 and 9 post infection, mice were administered 1.5 mg of A32 or
499 3BNC117 (WT or GRLR) mAb subcutaneously (S.C.). (B) Mice were bled routinely for
500 plasma viral load (PVL) and flow cytometry analysis. PVL levels were measured by
501 quantitative real-time PCR (limit of detection = 300 copies/mL, dotted line). (C) Percentage
502 of CD4+ T cells in peripheral blood was evaluated by flow cytometry . At least six mice
503 were used for each treatment. (D-E) Tissues of JRCSF-infected NSG-15 hu-PBL mice,
504 treated or not with A32 or 3BNC117 (WT or GRLR) were harvested at day 11. (D)
505 Percentage of CD4+ T cells was evaluated by flow cytometry (E) CD4+ T cells were
506 isolated for real-time PCR analysis of HIV DNA. Each dot represents the mean values +/-
507 SEM. S.C., subcutaneous; I.P., intraperitoneal; BM, bone marrow; mock-treated = no
508 antibody. Statistical significance was tested using one way ANOVA with a Holm-Sidak
509 post-test or a Kruskal-Wallis test with a Dunn's post-test (* p<0.05,** p<0.01,*** p<0.001)

510 **METHODS**

511

512 **Ethics Statement**

513 Written informed consent was obtained from all study participants and research adhered
514 to the ethical guidelines of CRCHUM and was reviewed and approved by the CRCHUM
515 institutional review board (ethics committee, approval number MP-02-2024-11734).
516 Research adhered to the standards indicated by the Declaration of Helsinki. All
517 participants were adult and provided informed written consent prior to enrolment in
518 accordance with Institutional Review Board approval.

519

520 **Cell lines and primary cells**

521 293T human embryonic kidney cells (obtained from ATCC) were maintained at 37°C under
522 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) (Wisent, St. Bruno, QC, Canada),
523 supplemented with 5% fetal bovine serum (FBS) (VWR, Radnor, PA, USA) and 100 U/mL
524 penicillin/streptomycin (Wisent). Human peripheral blood mononuclear cells (PBMCs)
525 from HIV-negative individuals and HIV-positive individuals obtained by leukapheresis and
526 Ficoll-Paque density gradient isolation were cryopreserved in liquid nitrogen until further
527 use. CD4+ T lymphocytes were purified from resting PBMCs by negative selection using
528 immunomagnetic beads per the manufacturer's instructions (StemCell Technologies,
529 Vancouver, BC) and were activated with phytohemagglutinin-L (10 µg/mL) for 48 h and
530 then maintained in RPMI 1640 (Thermo Fisher Scientific, Waltham, MA, USA) complete
531 medium supplemented with rIL-2 (100 U/mL).

532

533 **Antibody production**

534 FreeStyle 293F cells (Thermo Fisher Scientific) were grown in FreeStyle 293F medium
535 (Thermo Fisher Scientific) to a density of 1×10^6 cells/mL at 37°C with 8% CO_2 with regular

536 agitation (150 rpm). Cells were transfected with plasmids expressing the light and heavy
537 chains of each mAb using ExpiFectamine 293 transfection reagent, as directed by the
538 manufacturer (Thermo Fisher Scientific). One week later, the cells were pelleted and
539 discarded. The supernatants were filtered (0.22-μm-pore-size filter), and antibodies were
540 purified by protein A affinity columns, as directed by the manufacturer (Cytiva,
541 Marlborough, MA, USA).
542
543 **HIV-1 studies in humanized mice**
544 NSG-15 mice with expression of the human IL15 gene in the NOD/ShiLtJ background
545 were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The mice were bred
546 and maintained under specific pathogen-free conditions. All animal studies were
547 performed with authorization from Institutional Animal Care and Use Committees (IACUC)
548 of Yale University. NSG-15-Hu-PBL mice were engrafted as described (41). Briefly, 3.5 ×
549 10^6 PBMCs, purified by Ficoll density gradient centrifugation of healthy donor blood buffy
550 coats, obtained from the New York Blood Bank) were injected IP in a 200-μL volume into
551 6- to 8-week-old NSG-15 mice, using a 1-cm^3 syringe and 25-gauge needle. Cell
552 engraftment was tested 15 days post-transplant. 100 μL of blood was collected by
553 retroorbital bleeding. PBMCs were isolated by Ficoll density gradient centrifugation;
554 stained with fluorescently-labelled anti-human CD45 (BD Biosciences, Cat#: 555485) ,
555 CD3 (Biolegend, Cat#: 300424), CD4 (Biolegend, Cat#: 317432), CD8 (BD Biosciences,
556 Cat#: 561617) and CD56 (Biolegend, Cat#: 362508) antibodies and analyzed by flow
557 cytometry to confirm engraftment. Humanized mice were intraperitoneally challenged with
558 30,000 PFU of HIV-1_{JRCSF}. Infection profile was analyzed routinely by retro-orbital bleeding
559 and flow cytometric analysis of peripheral blood for human immune cells and PVL
560 analysis. For flow cytometry, 100 μl of blood was collected by retro-orbital bleeding at each
561 time point. PBMCs were isolated by Ficoll density gradient centrifugation and cells were

562 stained with fluor-conjugated antibodies as detailed above. PVL were measured at day 5,
563 8 and 11 post-infection, while HIV-1 reservoirs were measured at day 11 post-infected as
564 previously described (41).
565
566 **Plasmids and proviral constructs**
567 Transmitted/Founder (T/F) infectious molecular clone (IMC) of patient CH077 was inferred
568 and constructed as previously described (103). The generation of *nef*-defective CH077TF
569 was previously described and consists in the introduction of premature stop codons in the
570 *nef* reading frame using site-directed mutagenesis protocol (104). The CH077TF D368R
571 was also generated by site-directed mutagenesis as previously described (42). Proviral
572 constructs comprising an HIV-1 NL4.3-based isogenic backbone engineered for the
573 insertion of heterologous *env* strain sequences and expression in *cis* of full length Env
574 (Env-IMCs), were previously described (105). The Env-IMCs utilized in the present study
575 are those encoding Env form BaL (pNL-B.BaL.ecto), CH040 (pNL-B.CH040.ecto), CH058
576 (pNL-B.CH058.ecto), SF162 (pNL-B.SF162.ecto) or YU2 (pNL-B.YU-2.ecto). The proviral
577 plasmid pNL4.3 was used as control (106). The vesicular stomatitis virus G (VSV-G)-
578 encoding plasmid was previously described (107).
579
580 **Viral production, infections and *ex vivo* amplification.**
581 For *in vitro* infection, vesicular stomatitis virus G (VSV-G)-pseudotyped HIV-1 viruses
582 were produced by co-transfection of 293T cells with an HIV-1 proviral construct and a
583 VSV-G-encoding vector using the calcium phosphate method. Two days post-
584 transfection, cell supernatants were harvested, clarified by low-speed centrifugation (300
585 × g for 5 min), and concentrated by ultracentrifugation at 4°C (100,605 × g for 1 h) over
586 a 20% sucrose cushion. Pellets were resuspended in fresh RPMI, and aliquots were
587 stored at −80°C until use. Viruses were then used to infect activated primary CD4+ T cells

588 from healthy HIV-1 negative donors by spin infection at 800 × *g* for 1 h in 96-well plates
589 at 25 °C. Viral preparations were titrated directly on primary CD4+ T cells to achieve
590 similar levels of infection among the different IMCs tested (around 10% of p24+ cells). To
591 expand endogenously infected CD4+ T cells, primary CD4+ T cells obtained from PLWH
592 were isolated from PBMCs by negative selection. Purified CD4+ T cells were activated
593 with PHA-L at 10 µg/mL for 48 h and then cultured for at least 6 days in RPMI 1640
594 complete medium supplemented with rIL-2 (100 U/ml) to reach greater than 10% infection
595 for the ADCC assay.
596
597 **Antibodies**
598 The following anti-Env Abs were used to stained HIV-1-infected primary CD4+ T cells:
599 anti-gp41 ID F240; anti-cluster A A32, C11; anti-co-receptor binding site 17b; anti-gp120
600 outer domain 2G12, anti-CD4 binding site VRC03, 3BNC117; anti-V3 glycan PGT121,
601 PGT126, 10-1074; anti-V2 apex PG9; anti-gp120-gp41 interface PGT151. The HIV-IG
602 polyclonal antibody consists of anti-HIV immunoglobulins purified from a pool of plasma
603 from HIV+ asymptomatic donors (NIH AIDS Reagent Program). Goat anti-human IgG
604 (H+L) (Thermo Fisher Scientific) pre-coupled to Alexa Fluor 647 were used as secondary
605 antibodies in flow cytometry experiments. Rabbit antisera raised against Nef (NIH AIDS
606 Reagent Program) was used as primary antibodies in intracellular staining. BrillantViolet
607 421 (BV421)-conjugated donkey anti-rabbit antibodies (Biolegend) was used as
608 secondary antibodies to detect Nef antisera binding by flow cytometry. FITC or PE-
609 conjugated Mouse anti-human CD4 (clone OKT4; Biolegend) were used for cell-surface
610 staining of HIV-1-infected primary CD4+ T cells, while PE or FITC-conjugated Mouse anti-
611 HIV-1 p24 (clone KC57; Beckman coulter) were used for intracellular staining.
612
613 **Flow cytometry analysis of cell-surface staining**

614 Cell surface staining was performed at 48h post-infection. Mock-infected or HIV-1-infected
615 primary CD4+ T cells were incubated for 30 min at 37°C with anti-Env mAbs (5 µg/mL) or
616 HIV-IG (50 µg/mL). Cells were then washed once with PBS and stained with the
617 appropriate Alexa Fluor 647-conjugated secondary antibody (2 µg/mL) for 20 min at room
618 temperature. Cells were then stained with FITC- or PE-conjugated Mouse anti-CD4 Abs.
619 After two PBS washs, cells were fixed in a 2% PBS-formaldehyde solution. Infected cells
620 were then permeabilized using the Cytofix/Cytoperm Fixation/ Permeabilization Kit (BD
621 Biosciences, Mississauga, ON, Canada) and stained intracellularly using PE or FITC-
622 conjugated mouse anti-p24 mAb (clone KC57; Beckman Coulter, Brea, CA, USA; 1:100
623 dilution). The percentage of infected cells ($p24^{+}$) was determined by gating on the living
624 cell population according to a viability dye staining (Aqua Vivid; Thermo Fisher Scientific).
625 Alternatively, cells were stained intracellularly with rabbit antisera raised against Nef
626 (1:1000) followed by BV421-conjugated anti-rabbit secondary antibody. Samples were
627 acquired on an LSR II cytometer (BD Biosciences), and data analysis was performed
628 using FlowJo v10.5.3 (Tree Star, Ashland, OR, USA).
629
630 **Antibody-dependant cellular cytotoxicity (ADCC) assay**
631 Measurement of ADCC using a fluorescence-activated cell sorting (FACS)-based infected
632 cell elimination (ICE) assay was performed at 48 h post-infection. Briefly, HIV-1-infected
633 primary CD4+ T cells were stained with AquaVivid viability dye and cell proliferation dye
634 eFluor670 (Thermo Fisher Scientific) and used as target cells. Cryopreserved autologous
635 PBMC effectors cells, stained with cell proliferation dye eFluor450 (Thermo Fisher
636 Scientific), were added at an effector: target ratio of 10:1 in 96-well V-bottom plates
637 (Corning, Corning, NY). Anti-Env mAbs (5 µg/mL) was added to appropriate wells and
638 cells were incubated for 5 min at room temperature. The plates were subsequently

639 centrifuged for 1 min at 300 × g, and incubated at 37 °C, 5 % CO_2 for 5 h. before being
640 stained with FITC- or PE-conjugated Mouse anti-CD4 Abs. After one PBS wash, cells were
641 fixed in a 2% PBS-formaldehyde solution. Infected cells were then permeabilized using
642 the Cytofix/Cytoperm Fixation/ Permeabilization Kit (BD Biosciences, Mississauga, ON,
643 Canada) and stained intracellularly using PE or FITC-conjugated mouse anti-p24 mAb
644 (clone KC57; Beckman Coulter, Brea, CA, USA; 1:100 dilution) Productively-infected cells
645 were identified based on p24 and CD4 detection as described above. Samples were
646 acquired on an LSR II cytometer (BD Biosciences) and data analysis was performed using
647 FlowJo v10.5.3 (Tree Star). The percentage of ADCC was calculated with the following
648 formula: [(% of $CD4^+p24^{high}$ cells in Targets plus Effectors) − (% of $CD4^+p24^{high}$ cells in
649 Targets plus Effectors plus plasma or mAbs) / (% of $CD4^+p24^{high}$ cells in Targets) × 100]
650 by gating on infected lived target cells.
651
652 **RNAflow-FISH analysis.**
653 All buffers and fixation reagents were provided with the kit, with the exception of flow
654 cytometry staining buffer (2% FCS/PBS). The HIV-1 RNAflow-FISH assay was performed
655 as previously described and as per manufacturer's instructions (78-80). Briefly, cells were
656 harvested 48h post-infection and stained with the anti-Env mAbs A32 or PGT126 as
657 described above. Cells were then stained with Fixable Viability Dye (20 min, 4°C, Fixable
658 LiveDead, eBioscience) and then with a mix containing a brilliant stain buffer (BD
659 Biosciences) and the surface markers for CD4+ T cells detection (CD3 and CD4) and
660 CD8/NK/B cells and macrophages exclusions (CD8, CD56, CD19, CD16) (30 min, 4°C).
661 Samples were fixed, permeabilized with buffers provided by the manufacturer, and labeled
662 intracellularly for the structural HIV-1 p24 protein with the anti-p24 clone KC57 antibody
663 (30 min RT followed by 30 min 4°C, Beckman Coulter). HIV-1 RNA probing was performed

664 using the PrimeFlow RNA Assay (ThermoFisher). HIV-1 RNA were labeled using HIV-1
665 *env*RNA (Thermofisher; catalog number VF6-6000978) and HIV-1 *nef*RNA (Thermofisher;
666 catalog number (VF4-6000647) probe sets. HIV-1 *env*RNA was designed based on
667 CH077TF full env sequence, whereas HIV-1 *nef*RNA was based on consensus B HIV-1
668 sequence. Each probeset allows the hybridization of specific complementary branched
669 DNA nanostructure with different excitation/emission spectra. The probeset were diluted
670 1:5 in diluent and hybridized to the target mRNAs for 2 hr at 40°C. Samples were washed
671 to remove excess probes and stored overnight in the presence of RNAsin. Signal
672 amplification was achieved by performing sequential hybridization with DNA branches
673 (i.e., Pre-Amplifier and Amplifier) The first DNA branch in the Pre-Amplifier Mix was added
674 at a 1:1 ratio and was allowed to hybridize for 1.5 h at 40°C. Then the second DNA branch
675 in the Amplifier Mix was added and hybridized for 1.5 h at 40°C (78-80). Amplified mRNAs
676 were labeled with fluorescently tagged probes allowing hybridization for 1 hr at 40°C.
677 Samples were acquired on an LSRFortessa (BD Biosciences) and analyzed using FlowJo
678 (BD, V10.7.0). Unspecific binding of the fluorescent labeled branched probe in the
679 multiplex kit can lead to a low level of false-positive background noise, which, if present,
680 is detected across all the four channels corresponding to the types of labeled probes
681 (AF488, AF594, AF647, AF750). To decrease background noise, we thus left the AF594
682 channel vacant and excluded false-positive events based on fluorescence in this channel
683 before further gating. Gates were set on the HIV-uninfected donor control, or unstimulated
684 control where appropriate.
685

686 **QUANTIFICATION AND STATISTICAL ANALYSIS**

687 Statistics were analyzed using GraphPad Prism version 9.1.0 (GraphPad, San Diego, CA,
688 USA). Every data set was tested for statistical normality and this information was used to
689 apply the appropriate (parametric or nonparametric) statistical test. P values <0.05 were

690 considered significant; significance values are indicated as * $P<0.05$, ** $P<0.01$, ***
691 $P<0.001$, **** $P<0.0001$.
692
693 **Data availability**
694 The published article includes all datasets generated and analyzed for this study. Further
695 information and requests for resources and reagents should be directed to and will be
696 fulfilled by the Lead Contact Author (andres.finzi@umontreal.ca). All unique reagents
697 generated in this study are available from the Lead Contact with a completed Materials
698 Transfer Agreement.

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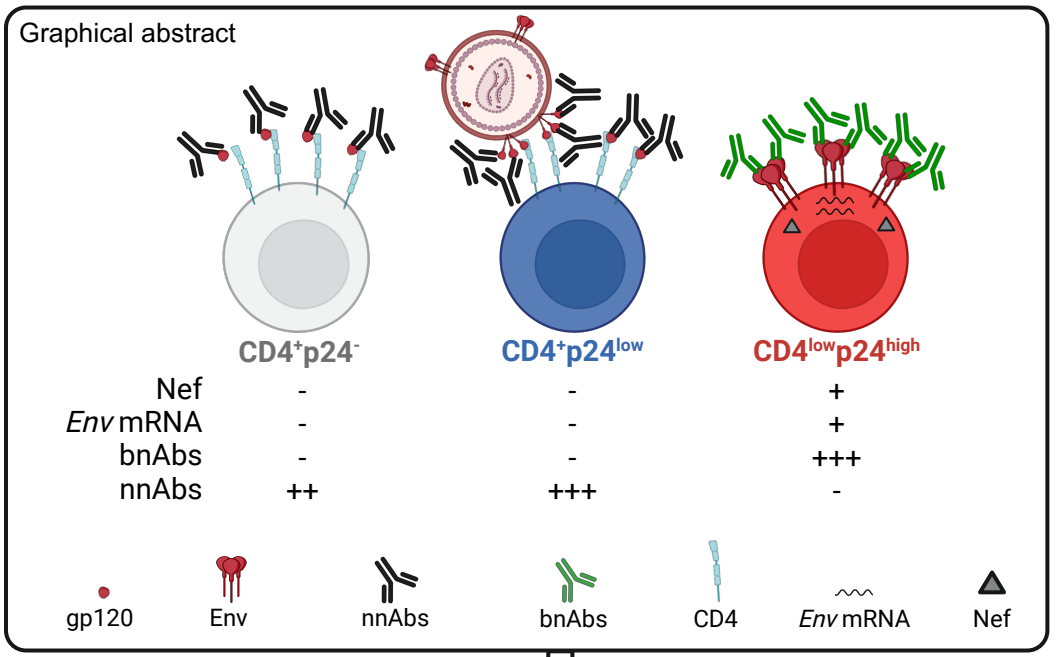
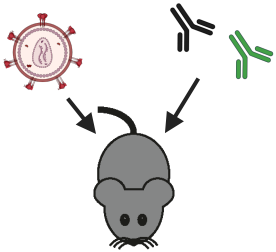
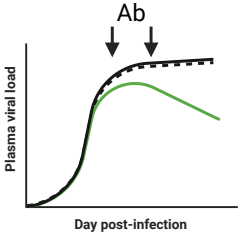
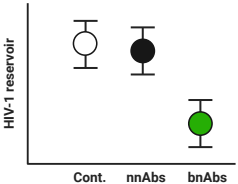
Graphical abstract









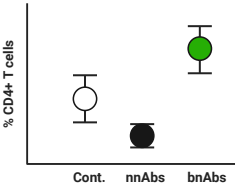


Figure 1

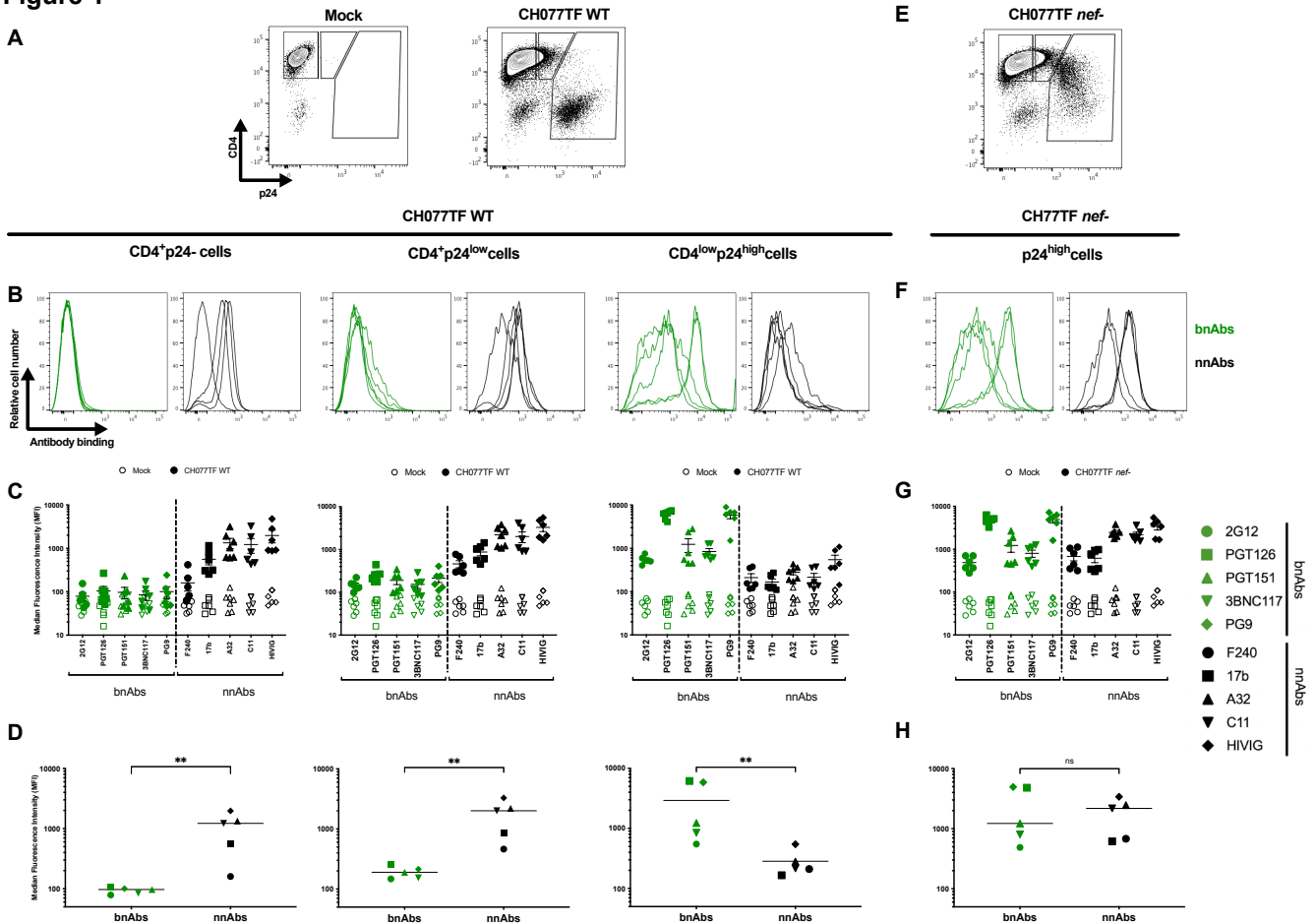
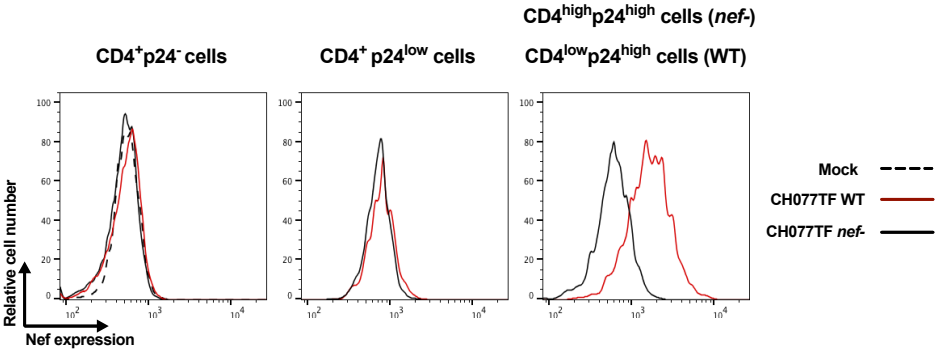

A
Mock
CH077TF WT
CD4
p24
E
CH077TF nef-
CH077TF WT
CH77TF nef-
CD4+p24- cells
CD4+p24low cells
CD4lowp24high cells
p24high cells
B
Relative cell number
Antibody binding
F
bnAbs
nnAbs
Mock
CH077TF WT
CH077TF nef-
C
G
Median Fluorescence Intensity (MFI)
2G12
PGT126
PGT151
3BNC117
PG9
F240
17b
A32
C11
HIVIG
bnAbs
nnAbs
D
H
**
ns

Figure 2

A


CD4+p24- cells
CD4+ p24low cells
CD4highp24high cells (nef-)
CD4lowp24high cells (WT)
Relative cell number
Nef expression
Mock
CH077TF WT
CH077TF nef-

B

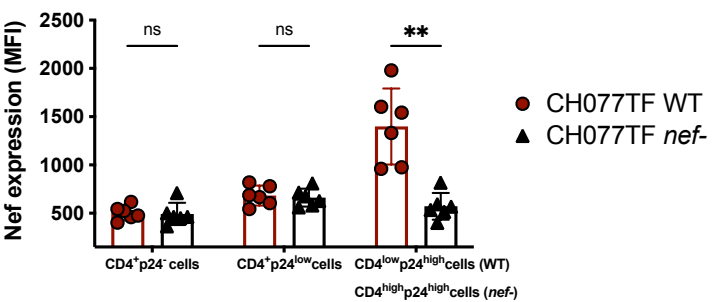

Nef expression (MFI)
ns
ns
**
CD4+p24- cells
CD4+p24lowcells
CD4lowp24highcells (WT)
CD4highp24highcells (nef-)
CH077TF WT
CH077TF nef-

Figure 3

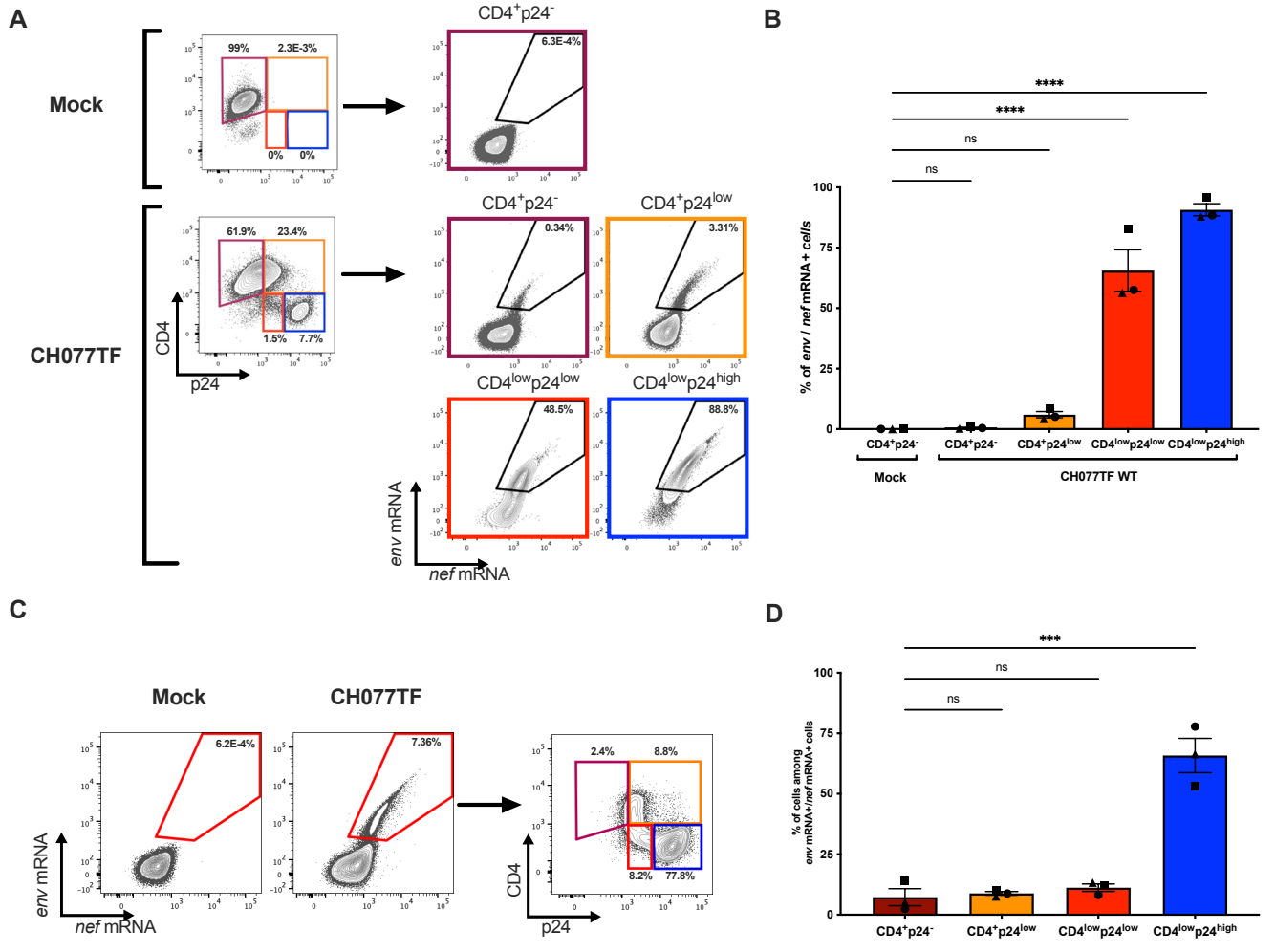

A
Mock
CH077TF
99%
2.3E-3%
0%
0%
61.9%
23.4%
1.5%
7.7%
CD4
p24
CD4+p24-
6.3E-4%
CD4+p24-
0.34%
CD4+p24low
3.31%
CD4lowp24low
48.5%
CD4lowp24high
88.8%
env mRNA
nef mRNA
B
****
****
ns
ns
% of env / nef mRNA+ cells
100
75
50
25
0
CD4+p24-
CD4+p24-
CD4+p24low
CD4lowp24low
CD4lowp24high
Mock
CH077TF WT
C
Mock
CH077TF
6.2E-4%
7.36%
env mRNA
nef mRNA
2.4%
8.8%
8.2%
77.8%
CD4
p24
D
***
ns
ns
% of cells among
env mRNA+/nef mRNA+ cells
100
75
50
25
0
CD4+p24-
CD4+p24low
CD4lowp24low
CD4lowp24high

Figure 4

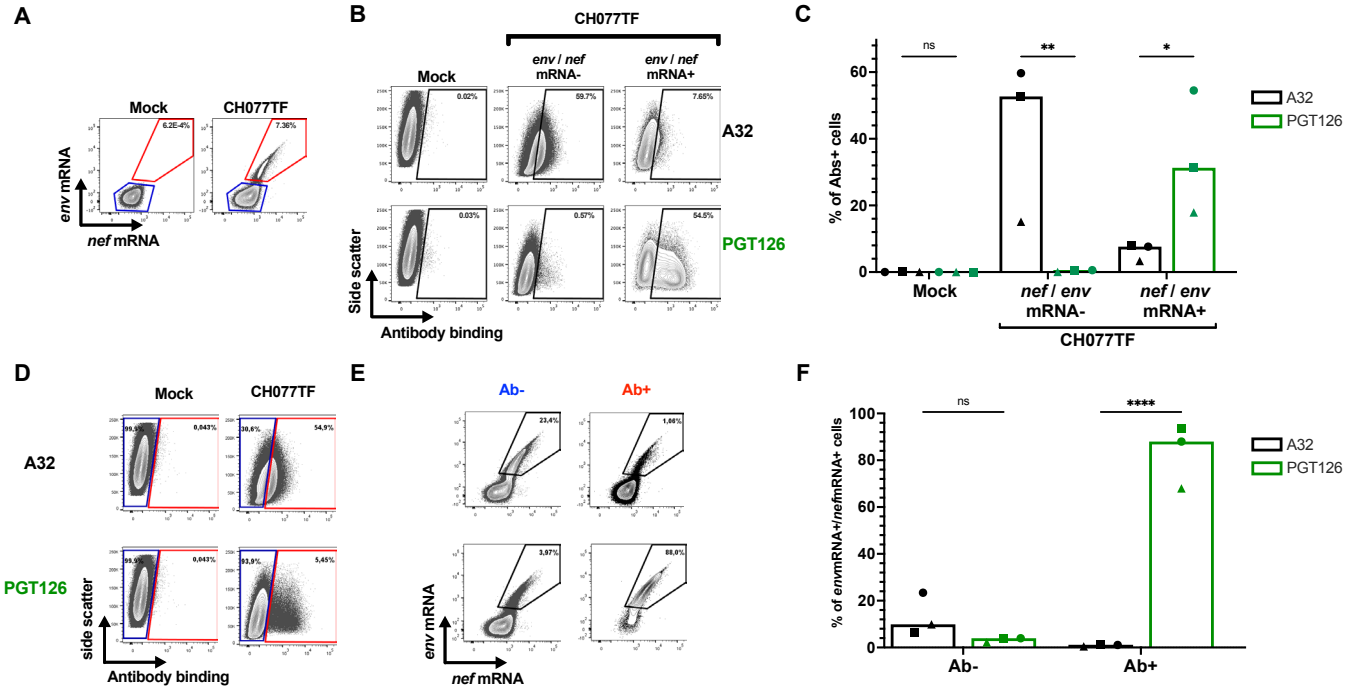

A
Mock
CH077TF
6.2E-4%
7.36%
env mRNA
nef mRNA
B
CH077TF
Mock
env / nef mRNA-
env / nef mRNA+
0.02%
59.7%
7.65%
A32
0.03%
0.57%
54.5%
PGT126
Side scatter
Antibody binding
C
ns
**
*
% of Abs+ cells
60
40
20
0
A32
PGT126
Mock
nef / env mRNA-
nef / env mRNA+
CH077TF
D
Mock
CH077TF
A32
99,9%
0,043%
30,6%
54,9%
PGT126
99,9%
0,043%
93,9%
5,45%
side scatter
Antibody binding
E
Ab-
Ab+
23,4%
1,06%
3,97%
88,0%
env mRNA
nef mRNA
F
ns
****
% of envmRNA+/nefmRNA+ cells
100
80
60
40
20
0
A32
PGT126
Ab-
Ab+

Figure 5

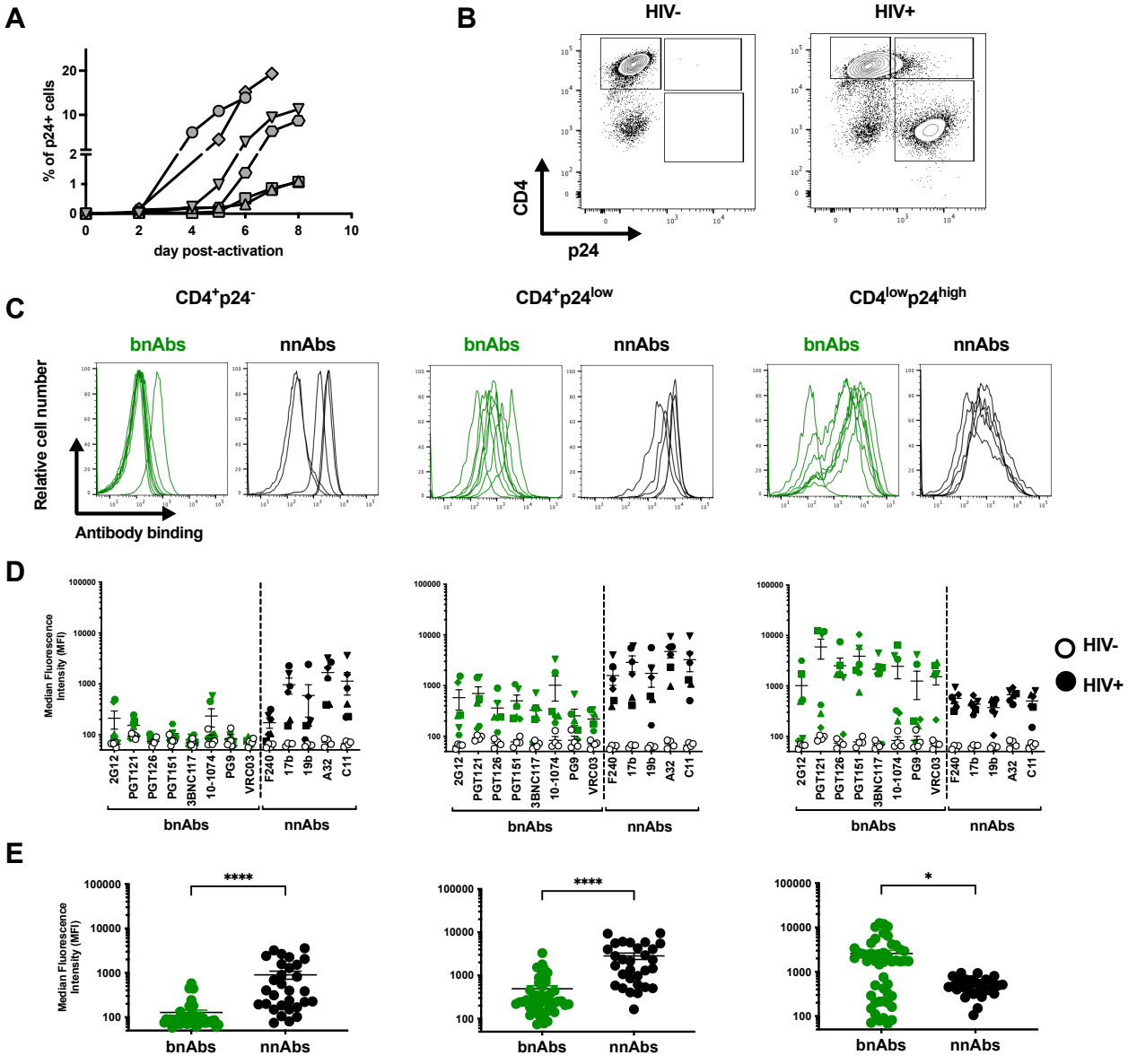

A
% of p24+ cells
day post-activation
B
HIV-
HIV+
CD4
p24
C
CD4+p24-
CD4+p24low
CD4lowp24high
bnAbs
nnAbs
Relative cell number
Antibody binding
D
Median Fluorescence Intensity (MFI)
2G12
PGT121
PGT126
PGT151
3BNC117
10-1074
PG9
VRC03
F240
17b
19b
A32
C11
bnAbs
nnAbs
HIV-
HIV+
E
Median Fluorescence Intensity (MFI)
****
*

Figure 6

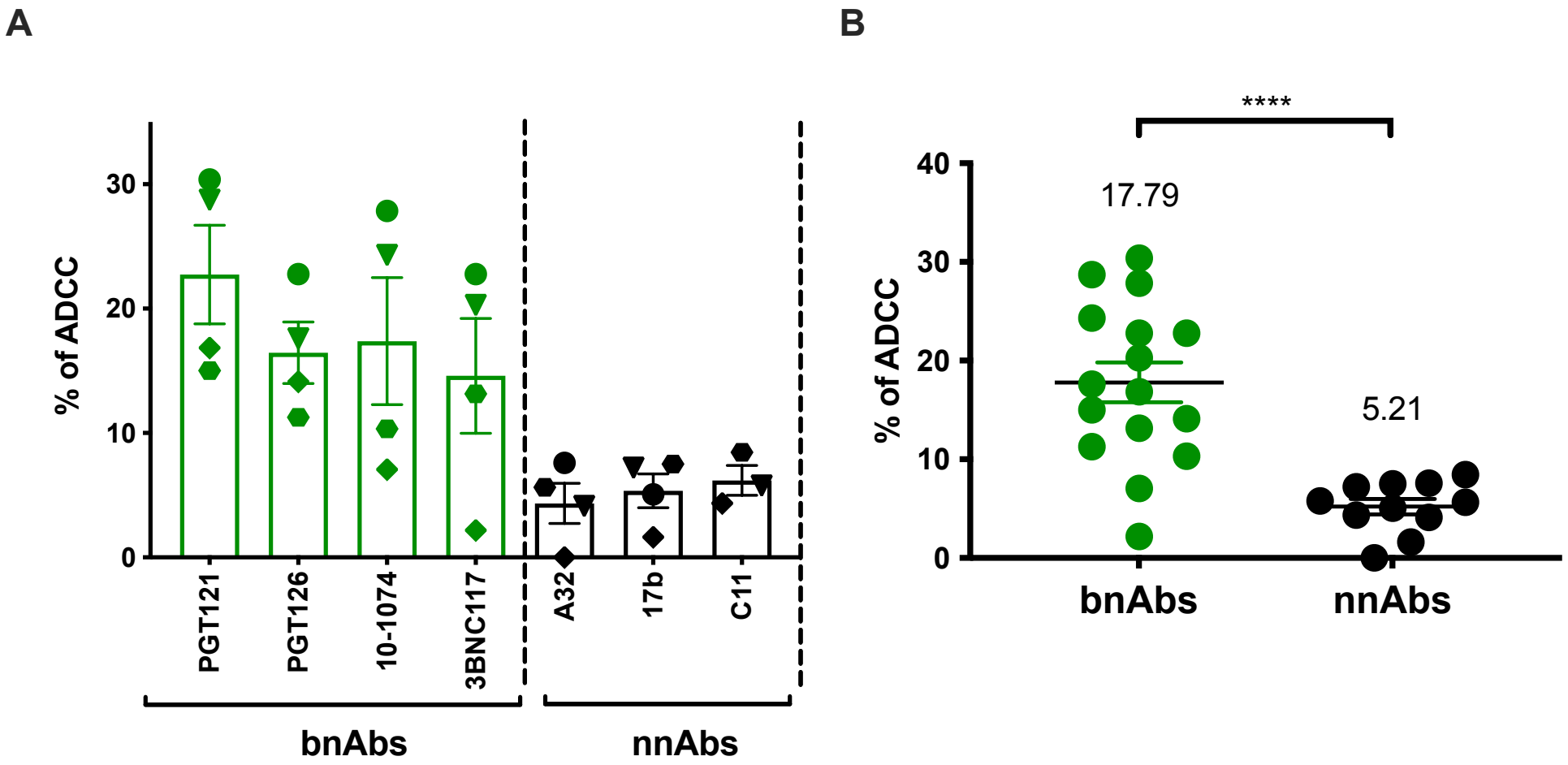
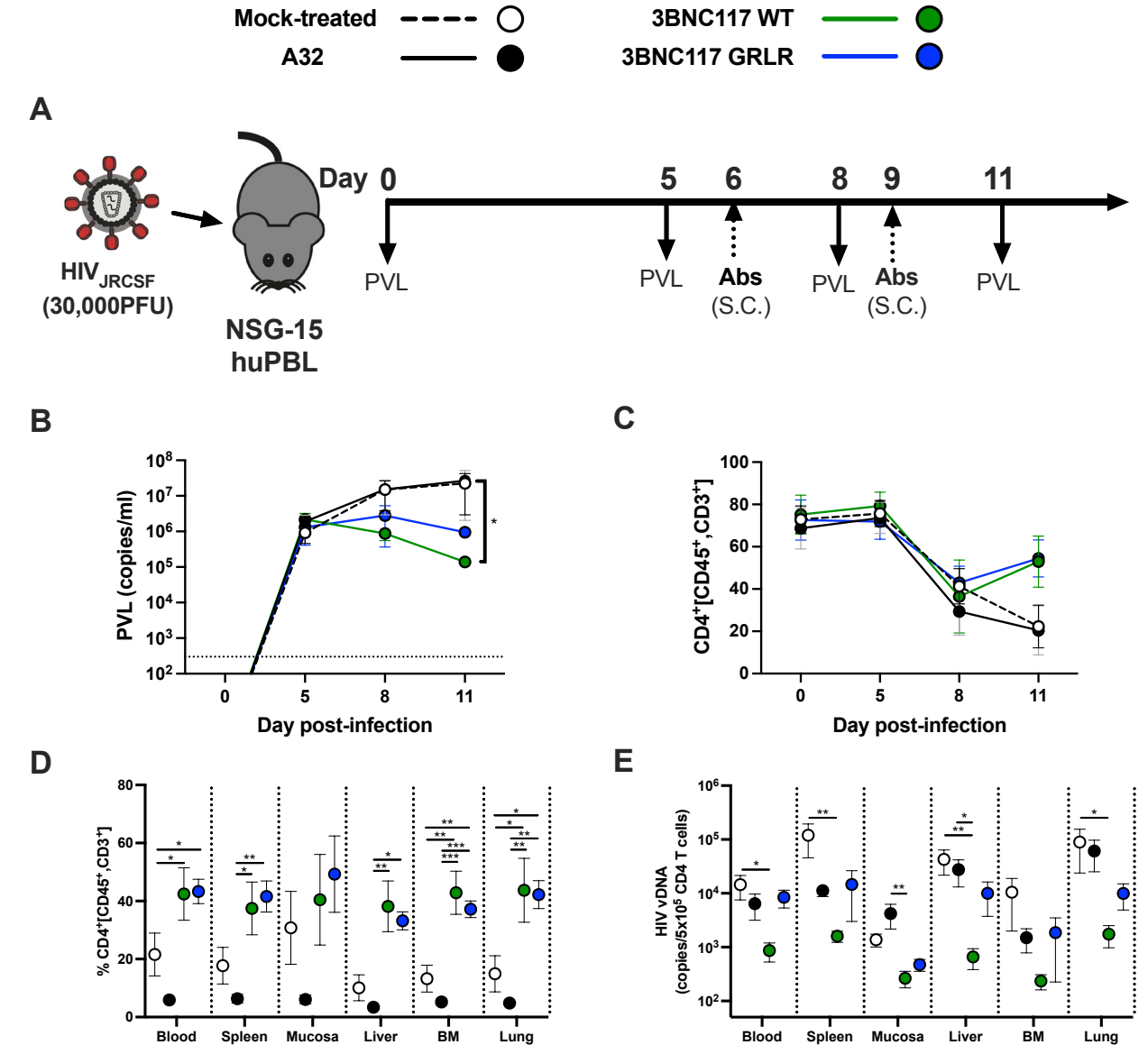

A
% of ADCC
30
20
10
0
PGT121
PGT126
10-1074
3BNC117
A32
17b
C11
bnAbs
nnAbs
B
****
% of ADCC
40
30
20
10
0
17.79
5.21
bnAbs
nnAbs

Figure 7


Mock-treated
A32
3BNC117 WT
3BNC117 GRLR
A
HIV JRCSF
(30,000PFU)
NSG-15
huPBL
Day 0
5
6
8
9
11
PVL
PVL
Abs
(S.C.)
PVL
Abs
(S.C.)
PVL
B
PVL (copies/ml)
Day post-infection
C
CD4+[CD45+,CD3+]
Day post-infection
D
% CD4+[CD45+,CD3+]
Blood
Spleen
Mucosa
Liver
BM
Lung
E
HIV vDNA
(copies/5x10^5 CD4 T cells)
Blood
Spleen
Mucosa
Liver
BM
Lung

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Impact of the D368R mutation on nnAbs and bnAbs binding.

Primary CD4+ T cells, mock-infected or infected with the transmitted-founder virus CH077, either expressing the wild-type (WT) or D368R Env (D368R) were stained with a panel of bnAbs and nnAbs, followed with appropriate secondary Abs. Cells were then stained for cell-surface CD4 prior detection of intracellular HIV-1 p24. (A) Graphs shown represent the median fluorescence intensities (MFI) obtained for at least 3 independent staining with the different mAbs. Error bars indicate means ± standard errors of the means. (B) Graphs shown represent the mean MFI obtained with each mAbs. (C) Percentage of the different cell populations at 48h post-infection. Statistical significance was tested using Mann-Whitney U test (** $p<0.01$, ns: non-significant).

Figure S2. Recognition of cells infected with HIV-1 constructs expressing lab-adapted and primary Envs by bnAbs and nnAbs.

Primary CD4+ T cells, mock-infected or infected with NL4.3 infectious molecular clones (IMC) expressing Env from lab-adapted (BaL, NL4.3) or primary (CH040TF, CH058, YU2, SF162) viruses were stained with a panel of bnAbs and nnAbs, followed with appropriate secondary Abs. Cells were then stained for cell-surface CD4 prior detection of intracellular HIV-1 p24. (A) Graphs shown represent the median fluorescence intensities (MFI) obtained for at least 3 independent staining with the different mAbs. Error bars indicate means ± standard errors of the means. (B) Graphs shown represent the mean MFI obtained with each mAbs for each HIV-1 IMC. (C-D) Percentage of the (C) $CD4^{low}p24^{high}$ and (D) $CD4^{+}p24^{low}$ populations at 48h post-infection. Statistical significance was tested Mann-Whitney U test (* $p<0.05$, ** $p<0.01$, **** $p<0.0001$).

Figure S3. CD4 downregulation precedes Env expression.

Primary CD4+ T cells, mock-infected or infected with the transmitted-founder virus CH077 WT were stained for cell-surface CD4 prior detection of intracellular HIV-1 p24 and *Env* mRNA and *nef* mRNA by RNA-flow FISH. (A) Example of RNA-flow FISH detection of *env* mRNA among the $CD4^{+}p24^{-}$, $CD4^{+}p24^{low}$, $CD4^{low}$ $p24^{low}$ or $CD4^{low}$ $p24^{high}$ cell populations. (C) Quantification of the percentage of *env* mRNA+ cells detected among the different cell population with three different donors. Statistical significance was tested using one way ANOVA with a Holm-Sidak post-test (* $p<0.05$, ** $p<0.01$, ns: non-significant).

Figure S4. Cells targeted by A32 are *env* mRNA negative.

Primary CD4+ T cells, mock-infected or infected with the transmitted-founder virus CH77 WT were stained with A32 or PGT126, followed with appropriate secondary Abs. Cells were then stained for cell-surface CD4 prior detection of intracellular HIV-1 p24 and *Env* mRNA and *nef* mRNA by RNA-flow FISH. (A) Histograms depicting representative *env* mRNA detection on A32+ or PGT126+ cells. (B) Median fluorescence intensities (MFI) of *env* mRNA obtained with 3 different donors. Statistical significance was tested using a Kruskal-Wallis test with a Dunn's post-test. (* $p<0.05$, ns: non-significant)

Figure S5. Nef expression in *ex vivo* expanded CD4+ T cells isolated from PLWH.

Ex vivo expanded CD4 T cells from 6 HIV+ individuals and 4 HIV- individuals were stained for surface CD4 prior to detection of intracellular Nef and p24. (A) Histograms depicting representative staining. (B) Median fluorescence intensities (MFI) obtained with 6 HIV+ individuals and 4 HIV- individuals. Statistical significance was tested using one-way ANOVA test with a Holm-Sidak post-test (**** $p<0.0001$, ns: non-significant).

Figure S6. A32 reduces the levels of CD4+ T cells in tissues *in vivo*. NSG-15-Hu-PBL mice were infected with HIV-1 JRCSF intraperitoneally. At day 6 and 9 post infection, mice were administered 1.5 mg of A32 mAb subcutaneously (s.c.). The percentage of CD4+ T cells was evaluated by flow cytometry in blood, spleen, mucosa, liver, bone marrow (BM) and lung at day 11 post-infection. Statistical significance was tested using a Mann Whitney U test (** $p<0.01$).

Figure S1

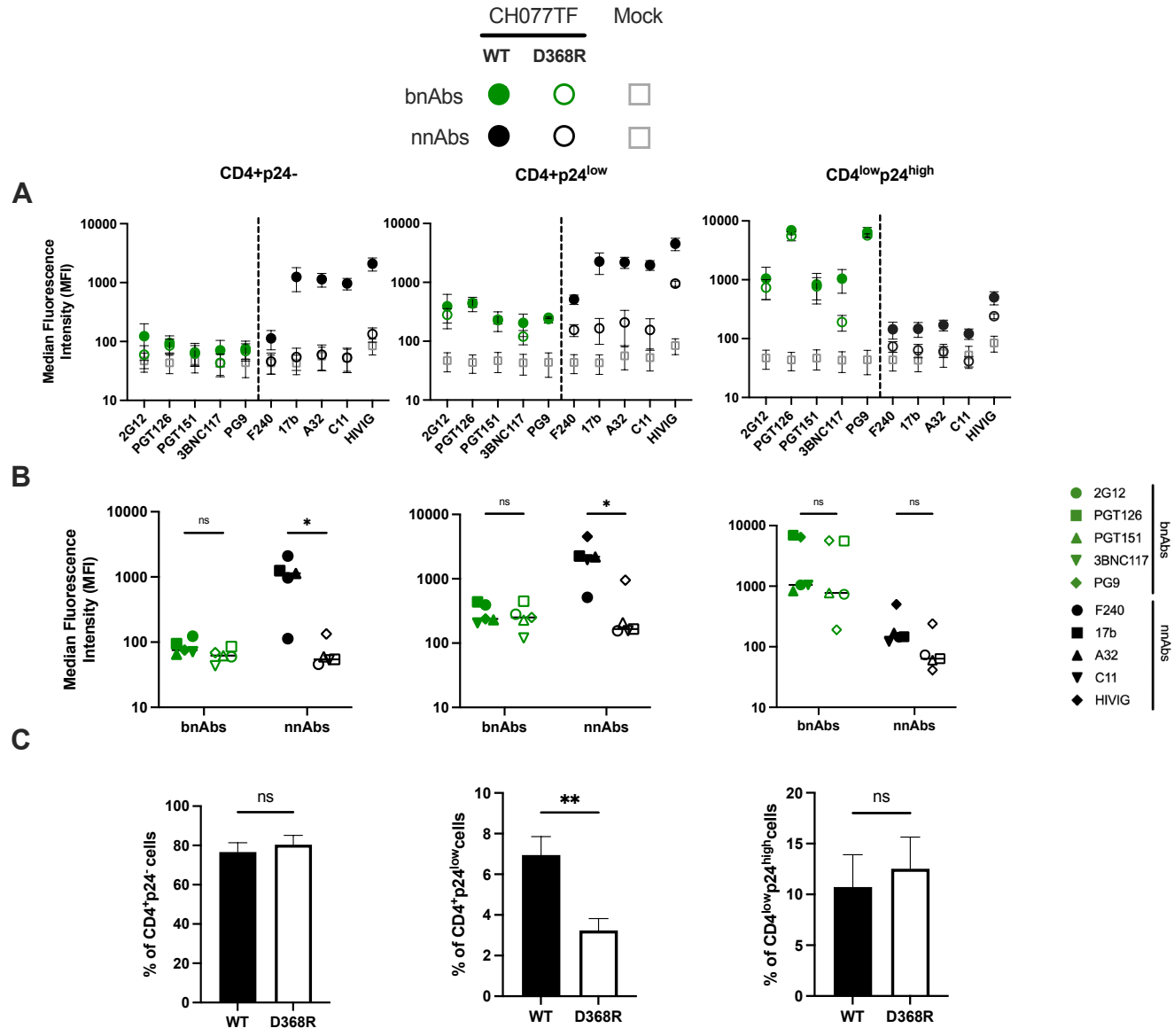

CH077TF
Mock
WT
D368R
bnAbs
nnAbs
A
CD4+p24-
CD4+p24low
CD4lowp24high
Median Fluorescence Intensity (MFI)
10000
1000
100
10
2G12
PGT126
PGT151
3BNC117
PG9
F240
17b
A32
C11
HIVIG
B
ns
*
bnAbs
nnAbs
2G12
PGT126
PGT151
3BNC117
PG9
F240
17b
A32
C11
HIVIG
C
% of CD4+p24- cells
% of CD4+p24low cells
% of CD4lowp24high cells
**
WT
D368R

Figure S2

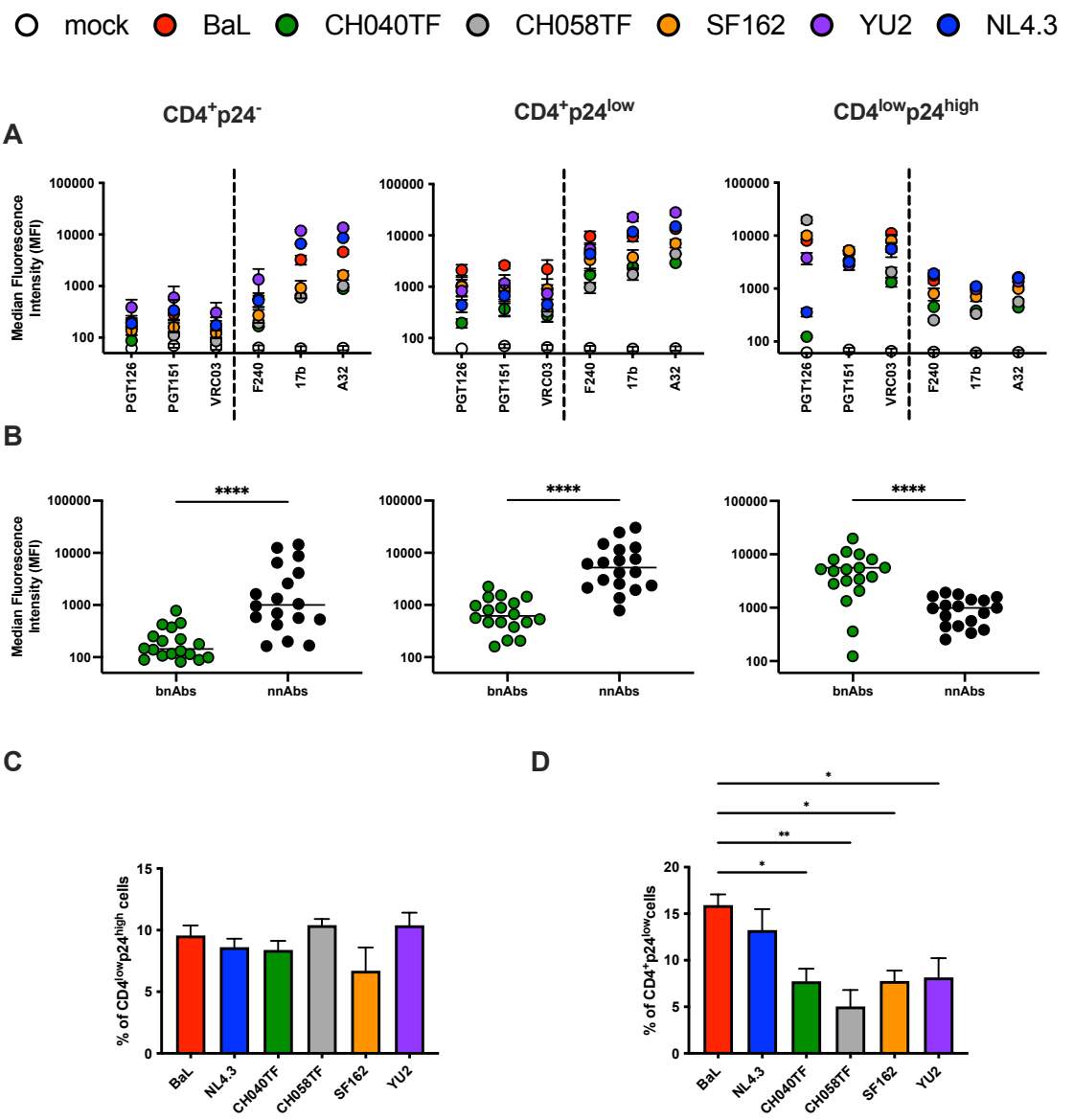
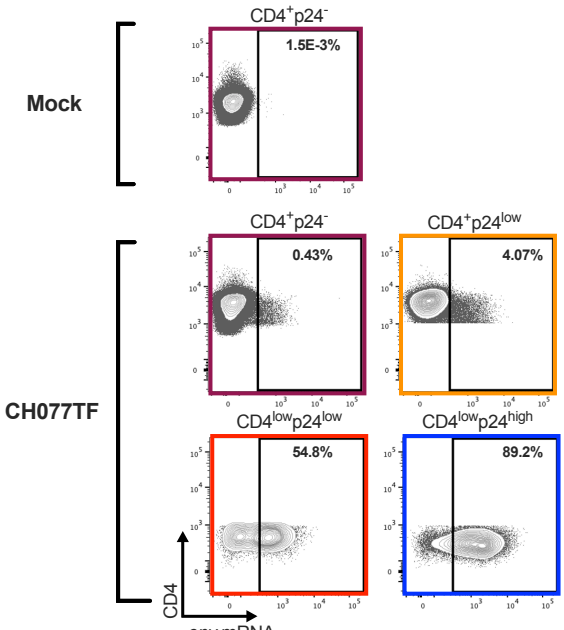

mock
BaL
CH040TF
CH058TF
SF162
YU2
NL4.3
A
CD4+p24-
CD4+p24low
CD4lowp24high
Median Fluorescence Intensity (MFI)
100000
10000
1000
100
PGT126
PGT151
VRC03
F240
17b
A32
B
****
bnAbs
nnAbs
C
% of CD4lowp24high cells
15
10
5
0
BaL
NL4.3
CH040TF
CH058TF
SF162
YU2
D
*
**
% of CD4+p24low cells
20

Figure S3

A


Mock
CH077TF
CD4+p24-
1.5E-3%
CD4+p24-
0.43%
CD4+p24low
4.07%
CD4lowp24low
54.8%
CD4lowp24high
89.2%
CD4
env mRNA

B

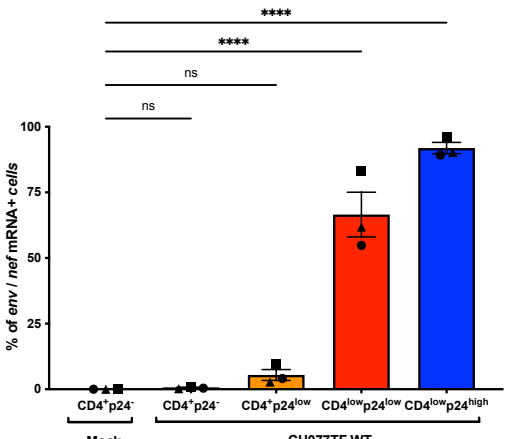
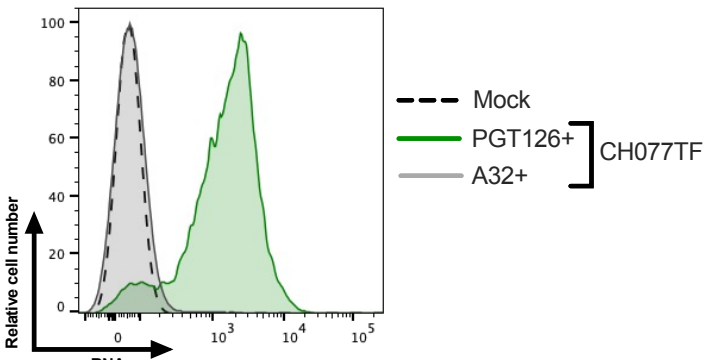

****
****
ns
ns
% of env / nef mRNA+ cells
100
75
50
25
0
CD4+p24-
CD4+p24-
CD4+p24low
CD4lowp24low
CD4lowp24high
Mock
CH077TF WT

Figure S4

A


100
80
60
40
20
0
Relative cell number
0
10^3
10^4
10^5
env mRNA
Mock
PGT126+
A32+
CH077TF

B

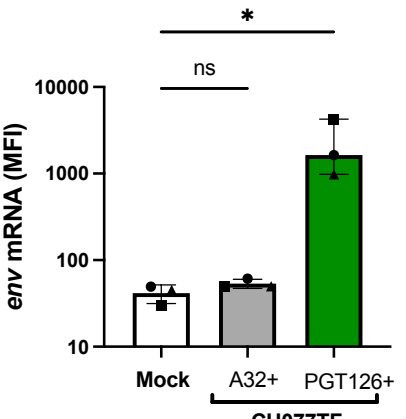
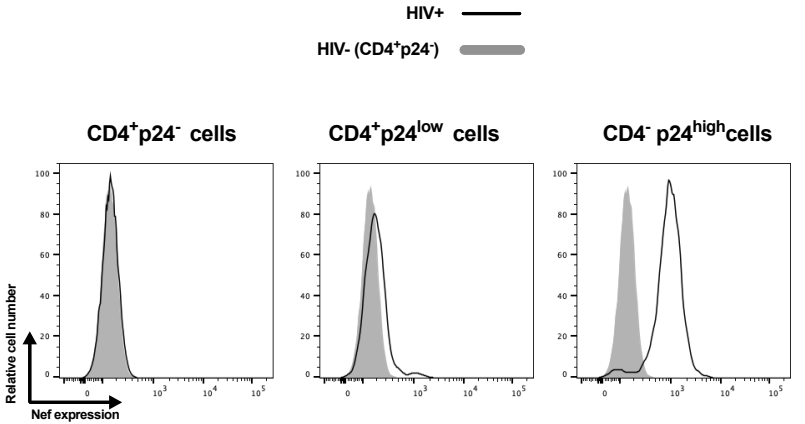

*
ns
10000
1000
100
10
env mRNA (MFI)
Mock
A32+
PGT126+
CH077TF

Figure S5


HIV+
HIV- (CD4+p24-)
A
CD4+p24- cells
CD4+p24low cells
CD4- p24high cells
Relative cell number
Nef expression

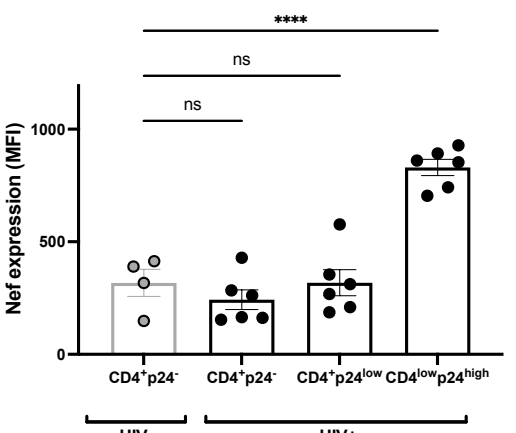
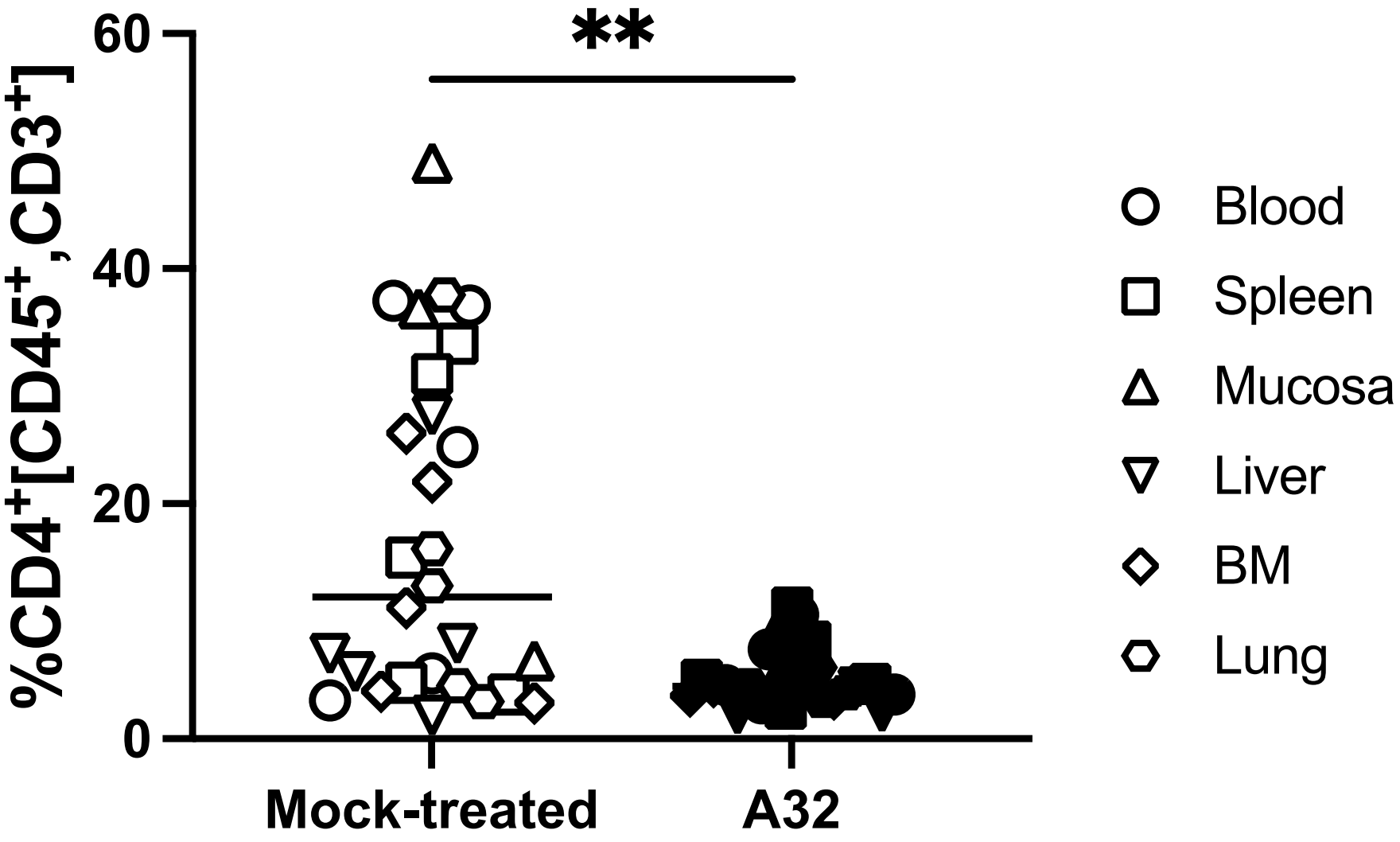

B
****
ns
ns
Nef expression (MFI)
1000
500
0
CD4+p24-
CD4+p24-
CD4+p24low
CD4lowp24high
HIV-
HIV+

Figure S6


**
60
40
20
0
%CD4+[CD45+,CD3+]
Mock-treated
A32
Blood
Spleen
Mucosa
Liver
BM
Lung