

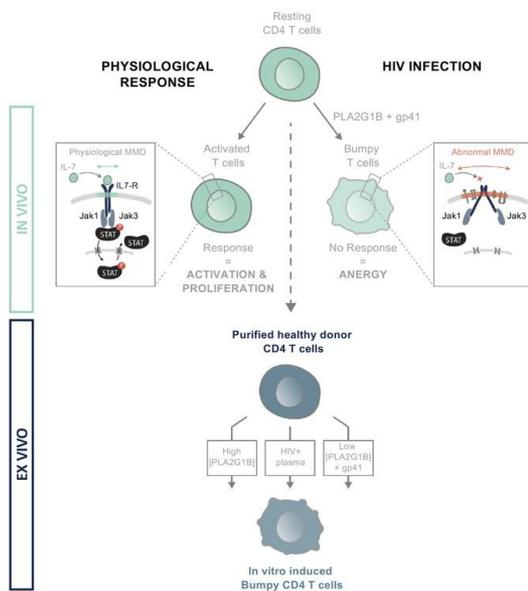
PLA2G1B is involved in CD4 anergy and CD4 lymphopenia in HIV-infected patients

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1 **TITLE: PLA2G1B IS INVOLVED IN CD4 ANERGY AND CD4**
2 **LYMPHOPENIA IN HIV-INFECTED PATIENTS**

3

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26

27 **CONFLICT OF INTEREST**

28 J.T. is cofounder and CEO of DIACCURATE, a spin-off of the Institut Pasteur. J.P., L.J. and

29 A.M. are employees of DIACCURATE.

30 **ABSTRACT**

31 The precise mechanism leading to profound immunodeficiency of HIV-infected patients is still
32 only partially understood. Here, we show that more than 80% of CD4 T cells from HIV-infected
33 patients have morphological abnormalities. Their membranes exhibited numerous large
34 abnormal membrane microdomains (aMMDs), which trap and inactivate physiological
35 receptors, such as that for IL-7. In patient plasma, we identified phospholipase A2 group IB
36 (PLA2G1B) as the key molecule responsible for the formation of aMMDs. At physiological
37 concentrations, PLA2G1B synergized with the HIV gp41 envelope protein, which appears to
38 be a driver that targets PLA2G1B to the CD4 T-cell surface. The PLA2G1B/gp41 pair induced
39 CD4 T cell unresponsiveness (anergy). At high concentrations in vitro, PLA2G1B acted alone,
40 independently of gp41, and inhibited the IL-2, IL-4, and IL-7 responses, as well as TCR-
41 mediated activation and proliferation, of CD4 T cells. PLA2G1B also decreased CD4 T-cell
42 survival in vitro, likely playing a role in CD4 lymphopenia in conjunction with its induced IL-
43 7 receptor defects. The effects on CD4 T-cell anergy could be blocked by a PLA2G1B-specific
44 neutralizing mAb in vitro and in vivo. The PLA2G1B/gp41 pair constitutes a new mechanism
45 of immune dysfunction and a compelling target for boosting immune responses in HIV-infected
46 patients.

47 **INTRODUCTION**

48 CD4 lymphocytes play a critical role in the severe immunodeficiency that characterizes HIV-
49 infected patients. Although less than 0.5% of blood CD4 T cells are infected, almost all are
50 dysfunctional. Their progressive decline leads to CD4 lymphopenia. In addition, functional
51 defects of the remaining CD4 T cells lead to their unresponsiveness, or anergy, to certain
52 antigens and cytokines (1). Major progress has been made in treatment of the viral infection.
53 Antiretrovirals (ARVs) prevent acquired immunodeficiency syndrome (AIDS). However,
54 further improvement in HIV therapy will require a better understanding of the mechanisms
55 responsible for CD4 T-cell defects following HIV infection (2, 3).

56 The mechanism explaining CD4 T-cell loss during HIV infection is still debated (4, 5).
57 Persistent immune activation plays a critical role in the induction of this CD4 T-cell decline (6–
58 8). A major mechanism results from damage of the mucosal barrier and lymphoid tissues of the
59 gastrointestinal (GI) track that follows acute infection. HIV targets subpopulations of CCR5-
60 expressing CD4 T cells, which are dense in the GI. Following this damage, microbial products
61 translocate across the GI barrier and cause general activation of the immune system (9–11). In
62 this context, it is noteworthy that HIV controller patients, who maintain high CD4 counts and
63 good control of viremia, show low inflammation (12).

64 Numerous investigations have described the impairment of CD4 T-cell function in HIV-
65 infected individuals, in whom CD4 T cells fail to proliferate after stimulation by antigens or
66 mitogens (13, 14). A progressive loss of T helper function has also been reported (15–17).
67 These results may be partially explained by changes in the T-cell receptor repertoire (18), but
68 they may also result from a defect in the intrinsic capacity of the CD4 T cells to respond to
69 physiological signals. For example, a selective defect in IL-2 production, but not
70 IFN γ synthesis, has been reported after anti-CD3 stimulation (19). In this context, we

71 previously analyzed CD4 T cell responses to IL-2 and IL-7, two cytokines that are crucial for
72 the control of the function, proliferation, and survival of CD4 T cells. We showed that the beta
73 and gamma c (γ c) chains of IL-2 receptor (IL-2R) are under-expressed and non-functional, as
74 measured by reduced entry into the S+G2/M phases of the cell cycle (20). Similarly, decreased
75 expression of the IL-7R alpha chain (CD127) on the surface of CD4 T cells from HIV-infected
76 patients has been described and their function was shown to be defective (21). Altered induction
77 of the anti-apoptotic molecule Bcl-2 and decreased expression of CD25 after in vitro exposure
78 to IL-7 were measured (22). We subsequently showed that the Janus kinase (Jak)/Signal
79 Transducer and Activator of Transcription 5 (STAT5) signaling pathway is involved in these
80 defects (23, 24). Similar results involving γ c have been published by other investigators (25–
81 30).

82 Most of the studies performed in lymphocytes from HIV-infected patients have used FACS
83 analysis and in vitro functional immunological assays. Here, we reinvestigated this question by
84 studying purified CD4 T lymphocytes from viremic HIV-infected patients using imaging
85 techniques (31) and molecular approaches rarely used in this field (32–34). We detected large
86 abnormal membrane microdomains (aMMDs) at the surface of CD4 T cells purified from HIV-
87 infected patients in the absence of any stimulation. The aMMD-bearing cells were named
88 “Bumpy T cells”, due to their appearance after labeling. Their large aMMDs were shown to
89 trap all IL-7R chains (alpha and γ c). IL-7R chains lose their function when embedded in these
90 aMMDs. Consequently, the Jak/STAT pathway was not functional and IL-7-induced phospho-
91 STAT5 nuclear translocation (pSTAT5 NT) was inhibited. Bumpy T cells were recovered after
92 exposure of CD4 T cells from healthy donors (HD) to plasma from HIV-infected patients. After
93 characterization, we found that phospholipase A2 group IB (PLA2G1B) (35) was able to
94 recapitulate the effects of plasma from HIV-infected patients. It induced aMMDs and
95 consequently strongly affected numerous CD4 functions in vitro and in vivo (mouse model).

96 However, PLA2G1B was found to synergize with HIV gp41 envelope protein in the blood of
97 HIV-infected patients at physiological concentrations. Overall, our results provide new insights
98 into CD4 T-cell dysfunction and a mechanism for the CD4 anergy and lymphopenia observed
99 in chronically HIV-infected patients.

100 **RESULTS**

101 *Membrane alterations and signaling defects in CD4 T cells from HIV-infected patients.*

102 Stimulation emission depletion (STED) microscopy revealed numerous large aMMDs (up to
103 500/cell, with an average size > 200 nm) at the surface of purified CD4 T cells from VP, in the
104 absence of any activation. More than 80% of purified CD4 T cells, also called Bumpy T cells,
105 from all viremic patients (VPs) exhibited aMMDs (Figure 1, A-C). Under the same conditions,
106 resting CD4 T cells from healthy donors (HD) did not spontaneously exhibit any aMMDs,
107 whereas IL-7 stimulation promoted the formation of numerous physiological MMDs (pMMDs)
108 of smaller size (approximately 800/cell, with an average size of 100 nm) (Figure 1, A-C). In
109 contrast, IL-7 stimulation of CD4 T cells from VPs did not induce any observable changes in
110 their membranes (Figure 1, A and B).

111 We then examined the functional consequences of these morphological changes in the
112 membrane using the IL-7/IL-7R system as a readout. The function of IL-7Rs of VP CD4 T cells
113 was altered, as the IL-7-induced phosphorylation of STAT5 (pSTAT5) differed between CD4
114 T cells of HDs and VPs (Figure 1D); pSTAT5 nuclear translocation (pSTAT5 NT) was almost
115 completely abolished in the CD4 T cells from VPs after IL-7 stimulation. This resulted from
116 the difference in the kinetics of cytoplasmic phosphorylation of STAT5 and pSTAT5 NT
117 between CD4 T cells of VPs and HDs (Figure 1E).

118 We previously showed that IL-7-induced cytoskeletal organization is required for efficient
119 pSTAT5 NT in CD4 T cells of HDs and that colchicine and cytochalasin D treatment abolishes

120 pSTAT5 NT (34). These results are comparable to those obtained for non-treated VP CD4 T
121 cells (Supplemental Figure 1, A and B). Microfilaments and microtubules can be observed after
122 IL-7 stimulation. Due to the very small size of the cytoplasm of lymphocytes, these structures
123 were observed by pulsed STED microscopy. After staining by anti-tubulin antibodies,
124 microtubules could be observed in HD CD4 T cells but not VP CD4 T cells (Supplemental
125 Figure 1C). Similarly, microfilaments were visible in HD CD4 T cells after staining by anti-
126 actin antibodies but not in VP CD4 T cells (Supplemental Figure 1D). This further supports
127 that the IL-7/IL-7R system is nonfunctional in VP CD4 T cells.

128 Overall, these results confirm and structurally characterize the activation status of CD4 T
129 lymphocytes from VPs and provide a new insight into the mechanism of unresponsiveness of
130 these CD4 T cells.

131

132 *Biochemical analysis of aMMDs from the CD4 T lymphocytes of VPs.*

133 We further examined the mechanism linking the presence of aMMDs at the surface of the CD4
134 T lymphocytes from VPs and the loss of function of the IL-7/IL-7R system by performing a
135 biochemical analysis of their membranes. Cell lysates obtained after moderate detergent
136 treatment were examined on sucrose gradients. This technique allowed us to separate free
137 molecules which migrate in high-density fractions and the detergent-resistant membranes
138 (DRMs), structurally related to MMDs, which are recovered in the low-density fractions.
139 Flotillin-1 was found in both fractions and was used as a marker in these experiments (Figure
140 2A and Supplemental Material). IL-7R α chains and γ c chains were found as free molecules in
141 the high-density fractions of HD CD4 T-cell lysates. They were found in low-density DRMs
142 only after IL-7 stimulation (Figure 2A). In contrast, IL-7R α chains and γ c chains were
143 spontaneously found associated with the low-density DRMs in lysates prepared from the
144 Bumpy T cells of VPs, in the absence of any stimulation (Figure 2A). IL-7R α appeared as

145 clusters in STED images of the CD4 T-cell membranes from VPs (Figure 2B), further
146 supporting the presence of low-density DRMs containing IL-7R chains. We further verified
147 that IL-7R α is included in aMMDs by studying its diffusion rate at the membrane surface. IL-
148 7R α was included in the aMMDs of VP CD4 T cells, as their diffusion was limited and could
149 be restored after disruption of the aMMDs by cholesterol oxidase and sphingomyelinase
150 treatment (Figure 2, C and D).

151 Overall these results demonstrate that IL-7R α and γc are spontaneously embedded in specific
152 macrostructures of the membranes of CD4 T cells from VPs, measured as aMMDs or DRMs.
153 These data also show that the receptors lose their function when trapped in this abnormal
154 structure of Bumpy CD4 T cells.

155

156 *Plasma from VPs induces the Bumpy T-cell phenotype in HD CD4 T cells.*

157 We investigated the molecular mechanism leading to the Bumpy T-cell phenotype. The addition
158 of plasma from VPs (Figure 3A) to HD CD4 T cells was sufficient to induce the Bumpy T-cell
159 phenotype. Titration showed the phenotype to be induced in 50% of the cells at approximately
160 1% VP plasma (Figure 3B). HD CD4 lymphocytes treated with VP plasma and Bumpy T cells
161 were microscopically undistinguishable, and the number and size of aMMDs at their surface
162 were not influenced by IL-7. In addition, plasma from elite controllers (HICp) (36, 37) and
163 patients with suppressed viremia after 10 years of ARV (ARTp) could not induce this
164 phenotype (Figure 3B).

165 We then studied the responsiveness of VP plasma-induced Bumpy T cells. IL-7-induced
166 pSTAT5 NT was inhibited by VP plasma, with a half maximum dose of 1% (Figure 3, C and
167 D). These results suggest a direct link between the induction of aMMDs and the mechanism
168 leading to the inhibition of pSTAT5 NT. We found a positive correlation between the number

169 of pMMDs and the frequency of cells with translocated pSTAT5 during IL-7 responses in HD
170 CD4 T cells (Figure 3E). Conversely, we found a negative correlation between the number of
171 aMMDs per CD4 T cell and the percent of cells with nuclear pSTAT5 in IL-7-stimulated
172 plasma-induced Bumpy T cells (Figure 3F). These correlations further support that plasma-
173 induced aMMDs are responsible for the loss of IL-7 response.

174

175 *Phospholipase A2 group IB (PLA2G1B) is a unique inducer of Bumpy T cells.*

176 We biochemically characterized the plasma molecule involved in these morphological and
177 functional changes. Size-exclusion and ion exchange chromatography were used to determine
178 the apparent MW and pI of the bioactive molecule(s) from the plasma of three VPs, using
179 microscopy as a read-out (Supplemental Figure 2, A-C). A list of 103 10-15 kDa proteins with
180 a pI between 6.5 and 7.5 and a secretory signal peptide was determined. Differential mass
181 spectrometry analysis identified PLA2G1B, also known as pancreatic phospholipase (35), as
182 the top candidate (PA21B in Supplemental Figure 2D). Active PLA2G1B is produced after the
183 cleavage of seven N-terminal residues from non-active proPLA2G1B (38). Recombinant
184 PLA2G1B was produced, purified, crystallized and structurally characterized. The position of
185 residues H48 and D99 and the Ca²⁺-binding loop, critical for the activity of the enzyme, are
186 shown in Figure 4A.

187 Recombinant PLA2G1B alone was able to induce aMMDs (Figure 4B) and inhibit pSTAT5
188 NT in HD CD4 T cells (Figure 4C). This property was catalytic site-dependent, as the non-
189 active H48Q mutant (39) was unable to induce aMMDs or inhibit pSTAT5 NT on human CD4
190 T cells (Figure 4, D and F). These effects were specific to PLA2G1B; indeed, other members
191 of the PLA2 family such as PLA2GIIA, PLA2GIID or PLA2GX showed no significant effect
192 on either aMMDs formation or pSTAT5 NT inhibition (Figure 4, E and G). Similarly, only
193 polyclonal antibodies specific for PLA2G1B decreased plasma-induced pSTAT5 NT

194 inhibition, whereas polyclonal antibodies specific for PLA2GIIA or PLA2GIID had no effect
195 (Figure 4H).

196 We developed mouse monoclonal antibodies (mAb) specific for PLA2G1B. Among them, mAb
197 14G9 efficiently inhibited the enzymatic activity of PLA2G1B and abrogated VP plasma-
198 inhibition of pSTAT5 NT in a dose-dependent manner (Figure 4I). These experiments show
199 that, at physiological concentrations, PLA2G1B is involved in the phenotypic and functional
200 changes induced by VP plasma in HD CD4 T cells and the Bumpy T-cell phenotype observed
201 in VP.

202

203 *PLA2G1B induces anergy of CD4 T cells: specificity and reversal of the effects.*

204 The unresponsiveness of CD4 T cells to IL-7 induced by PLA2G1B was also observed for IL-
205 2 and IL-4, two other γ c cytokines. Similar to IL-7, pSTAT NT-induced by these two cytokines
206 was inhibited by PLA2G1B in a dose-dependent manner and with a comparable IC50 (Figure
207 5A). These observations were verified using VP plasma-induced Bumpy T cells (Figure 5B).
208 Unlike IL-2, IL-4, and IL-7, IFN α -induced pSTAT1 NT was not inhibited by PLA2G1B or the
209 plasma of VPs (Figure 5, C and D). IFN- α is known to signal by a mechanism independent of
210 MMDs (40, 41). Thus, these results suggest that PLA2G1B only affects signaling pathways that
211 involve compartmentalization into pMMDs. We then studied the effects of PLA2G1B first
212 observed on total unseparated CD4 T cells, on naïve (CD45RA⁺) and memory (CD45RA⁻) CD4
213 T cells. PLA2G1B was slightly more active on CD45RA⁺ CD4 T cells than CD45RA⁻ CD4 T
214 cells (Figure 5E). Such differential sensitivity is not the consequence of selective modulation
215 of IL-7R (CD127) expression at the cell surface by PLA2G1B (Figure 5, F and G and
216 Supplemental Figure 3). As previously described, the percentage of CD127-positive cells was
217 slightly higher in CD45RA⁺ than CD45RA⁻ CD4 T cells (Figure 5F) (42, 43). In addition MFI
218 analysis of CD127 expression (Figure 5G and Supplemental Figure 3) showed a slight reduction

219 in CD45RA⁻ cells as previously reported (44). Overall, these analyses establish that PLA2G1B
220 does not influence CD127 expression and support our hypothesis that PLA2G1B acts on signal
221 transduction (as described above) and not by decreasing receptor expression.

222 The action of PLA2G1B appears to be specific to CD4 T cells. Indeed, PLA2G1B did not
223 induce aMMD formation in purified CD8 T cells from HDs (Figure 5H). Similarly, pSTAT5
224 NT was not affected in CD8 T cells by PLA2G1B, even at high concentrations (Figure 5I).
225 These results are consistent with *ex vivo* observations of VP CD8 T cells in which aMMDs
226 were not detectable and pSTAT5 NT continued to occur (Supplemental Figure 4, A-C). In
227 addition, physiological concentrations of PLA2G1B present in VP plasma inhibited pSTAT5
228 NT on CD4 T cells but had no functional effects in CD8 lymphocytes purified from HDs
229 (Supplemental Figure 4D).

230 PLA2G1B is known to digest lipids, we thus further explored the difference between the
231 response of CD4 and CD8 T cells to PLA2G1B by lipidomic analysis. There were significant
232 differences in the proportions of the ganglioside GM3, PC, PE, PI, PS, SM and TG between
233 HD CD4 and CD8 T cells (Supplemental Figure 4E). Similarly, differences in the lipid
234 proportions have been reported between murine CD4 and CD8 T cells (45). It is possible that
235 the differential effects of PLA2G1B on CD4 and CD8 T cells are associated with differences
236 in lipid composition, but direct evidence will require extensive studies.

237 We next investigated the reversal of the induced Bumpy T-cell phenotype *in vitro* and show the
238 results of one of three representative experiments (Figure 5J). Purified CD4 T cells were first
239 treated *in vitro* with PLA2G1B and then cultured for various periods of time up to 3 days.
240 Inhibition of pSTAT5 NT was examined every day. Under our experimental conditions, 30%
241 of the cells were anergized and did not respond to IL-7. After three days in culture, pSTAT5

242 NT returned to normal, clearly showing that the Bumpy T-cell phenotype is reversible.
243 Furthermore, neutralizing mAb 14G9 accelerated the reversion (Figure 5J).

244

245 *PLA2G1B affects CD4 T-cell survival in vitro: inhibition by neutralizing mAb 14G9.*

246 Aside from the unresponsiveness of CD4 T cells to physiological signals (anergy), HIV-
247 infected patients suffer from CD4 lymphopenia. We thus tested the effects of PLA2G1B on the
248 half-life of CD4 T cells in vitro. Purified CD4 lymphocytes were cultured and the number of
249 live cells counted over time as described in Methods. The number of surviving CD4 T cells
250 varied between donors in control cultures. The effects of PLA2G1B on the CD4 lymphocytes
251 are thus expressed as the percentage of the surviving cells relative to that in control cultures in
252 the absence of PLA2G1B at each time point. The effect of PLA2G1B on CD4 T-cell survival
253 was first tested at various concentrations up to 100 μ M (Figure 6A). We then verified that 50%
254 cells died after 18 days of culture in the presence of 1 μ M PLA2G1B (Supplemental Figure
255 5A) and 40% of the cells died after 24 days of culture in the presence of 250 nM PLA2G1B
256 (Figure 6B).

257 During these experiments, we further analyzed the CD4 T cells. As expected, numerous dying
258 cells become Annexin V-positive Zombie-positive. However, we also detected Annexin V-
259 negative Zombie-positive cells (Figure 6, C and D). Their percentage increased during culture,
260 reaching more than 70% of the recovered lymphocytes (Figure 6E). This was a specific
261 consequence of PLA2G1B treatment, as such cells were not detected when CD4 T cells were
262 cultured in the presence of the inactive PLA2G1B mutant H48Q (Figure 6C and Supplemental
263 Figure 5B). Similarly, their percentage was lower when cultures were performed in the presence
264 of mAb 14G9, which neutralizes the enzymatic activity of PLA2G1B (Figure 6, D and E). This
265 can be explained by the fact that PLA2G1B digested one of its substrates during culture,

266 phosphatidylserine, which is also the binding site of Annexin V. This confirms that the action
267 of PLA2G1B on CD4 T cells is mediated by its enzymatic activity.

268 We then tested the effect of mAb 14G9 on CD4 T-cell survival. The mAb significantly
269 increased the survival of CD4 T cells exposed to PLA2G1B (up to > 50%) relative to cultures
270 in the presence of the control isotype (Figure 6F and Supplemental Figure 5C).

271

272 *In vitro and in vivo effects of human PLA2G1B in a mouse model.*

273 We studied the effects of human PLA2G1B in mice after verifying its activity in vitro on mouse
274 CD4 T cells to extend our data in vivo. Upon exposure to PLA2G1B we also observed Annexin
275 V-negative Zombie-positive mouse CD4 T cells (Supplemental Figure 6). CD4 T cells from
276 C57BL/6 mice were purified from the spleen and stimulated by anti-CD3 plus anti-CD28 beads,
277 in the presence of IL-2. Human PLA2G1B was active on mouse CD4 T cells and induction of
278 CD25 (IL-2R α) was inhibited by PLA2G1B by day 5 in a dose-dependent manner (Figure 7,
279 A and B). Similarly, survival and proliferation were profoundly altered (Figure 7, C-E). These
280 effects depended on the catalytic activity of PLA2G1B, as the H48Q mutant was ineffective
281 (Figure 7, A-E). Furthermore, neutralizing mAb 14G9 blocked CD25 induction and decreased
282 survival of the CD4 cells (Figure 7, F and G). These data demonstrate that PLA2G1B can also
283 inhibit TCR responses. In addition to the human experiments, these data establish that the
284 effects of PLA2G1B can be measured after several days in culture.

285 In vivo, PLA2G1B showed activity on mouse CD4 lymphocytes in a dose-dependent manner
286 (Figure 7H). Injection of 100 μ g of PLA2G1B induced a long-lasting effect, which persisted
287 for up to 72 hours and began to diminish after 168 hours (Figure 7I). The effect of PLA2G1B
288 was maximal three hours after injection (Figure 7J). We tested the effects of pre-treatment with
289 the anti-PLA2G1B neutralizing mAb 14G9 under the same experimental conditions (Figure

290 7K). The blockade was close to 100%. We obtained a comparable result in a group of mice that
291 was pre-immunized against human PLA2G1B (Figure 7L).

292 The cell specificity of the effects of PLA2G1B was further verified in this experimental model.
293 Injection of PLA2G1B did not result in any loss of IL-7-induced pSTAT5 NT in mouse CD8
294 lymphocytes, as measured ex vivo three hours post-injection (Supplemental Figure 4F).
295 Overall, these results open the possibility of using mouse models to evaluate anti-PLA2G1B
296 neutralizing mAbs as an immunotherapeutic strategy.

297

298 *Synergy between PLA2G1B and plasma HIV gp41 protein.*

299 We then measured active and proPLA2G1B in the plasma of HD, HIC, ARV, and VP patients
300 using PLA2G1B ELISAs (Figure 8, A and B). VPs had similar levels of active PLA2G1B as
301 HIC and ART patients but slightly more active PLA2G1B than HDs (median increase of 1.4).
302 In addition, comparable copy numbers of *pla2g1b* RNA were found in the PBMCs of HDs and
303 VPs by qPCR (Figure 8C). We thought that these results cannot explain the difference in
304 PLA2G1B activity observed with the functional assays (Figure 3, B and D). This observation
305 led us to consider that one or more cofactors are present in the plasma of VPs and are required
306 for the induction of aMMDs and blockade of pSTAT5 NT. Indeed, the dose-response curve
307 (from 0.001 to 1,000 nM) of PLA2G1B diluted in HD or VP plasma previously depleted of
308 endogenous PLA2G1B showed striking differences in the IC50 values (Figure 8D), supporting
309 the presence of a cofactor. The IC50 of PLA2G1B was 75 nM when diluted in PBS buffer or
310 HD plasma, but decreased to 5 nM when diluted in PLA2G1B-depleted VP plasma. We
311 concluded that PLA2G1B was not acting alone but in synergy with another factor present in
312 VP plasma. A bioassay was developed, using a limiting amount of PLA2G1B inactive by itself
313 (5 nM, Figure 8D) and PLA2G1B-depleted VP plasma to detect the potential cofactor (Figure
314 8E), and used to show that the enhancement was lost after plasma was incubated with purified

315 CD4 T cells (Figure 8F), suggesting that the cofactor was adsorbed on CD4 T cells. After such
316 pretreatment, the addition of 5 nM PLA2G1B to the CD4 T cells, without further addition of
317 VP plasma, resulted in the inhibition of IL-7 driven pSTAT5 NT (Figure 8G). This cofactor
318 activity was sensitive to trypsin treatment and could be fractionated with an apparent MW
319 between 10 and 30 kDa. The search then focused on HIV peptides possibly released into the
320 plasma of infected patients. A gp41 fragment (MN 565-771 delta 642-725), and its
321 corresponding 3S peptide (46), of which the sequence is highly conserved among various HIV
322 isolates, exerted strong cofactor activity (Figure 8, H-K). In addition, the depletion of VP
323 plasma with anti-gp41-specific antibodies that do not bind to gp120 (Supplemental Figure 7)
324 resulted in the loss of cofactor activity (Figure 8L). This critical point was definitively
325 established after depletion by a mAb characterized in the laboratory. This mAb, 1C5, was raised
326 against the 3S peptide and shown to also recognize gp41 protein but not gp120 (Supplemental
327 Figure 7). It was also able to completely deplete various VP plasma samples of cofactor activity
328 (Figure 8M). These results support the hypothesis that fragments of gp41 containing the 3S
329 sequence may act as cofactors that target PLA2G1B to the surface of CD4 lymphocytes to exert
330 its enzymatic activity.

331

332 **DISCUSSION**

333 Here, we further characterize the activation status of the CD4 T-cell compartment in HIV-
334 infected patients. Aside from currently used cell-surface activation markers, such as CD38 and
335 HLA-DR, we show structural alterations of CD4 T-cell membranes, consisting of large aMMDs
336 observed at the cell surface. They arise due to the activity of the PLA2G1B enzyme, acting in
337 synergy with gp41. These findings support a new mechanism of immunosuppression in HIV-
338 infected patients. Here, we show that this mechanism is involved in CD4 anergy to γ c cytokine

339 and TCR responses. Furthermore, its action on the decreased survival of CD4 T cells suggests
340 a role in CD4 lymphopenia.

341 Bumpy T cells represent a new phenotype characterizing activated CD4 T cells found in HIV-
342 infected patients. It results from remodeling of the CD4 T-cell membrane under the influence
343 of the enzymatic activity of PLA2G1B. This exposes GM1 gangliosides, which are enriched in
344 aMMDs and recognized by labeled cholera toxin B using the STED imaging technique. Bumpy
345 T cells expressing numerous aMMDs up to 500/cell can be recovered from patient blood and
346 easily identified. In the blood of these individuals, aMMDs are spontaneously expressed by
347 CD4 T cells and their characteristic pattern is not modified by a strong stimulus, such as IL-7.
348 Given that this phenotype is observed in more than 80% of the CD4 T cells of HIV-infected
349 patients, a correlation with CD38 and HLA-DR activation markers should be investigated. In
350 any case, this observation offers new insights into our understanding of the dysfunction of the
351 CD4 compartment of HIV-infected patients.

352 At low concentrations, such as those found in the blood, PLA2G1B cannot act by itself. In HIV-
353 infected patients, it requires a cofactor, which we identified as a soluble fragment of gp41. Gp41
354 appears to be a driver that targets PLA2G1B to the CD4 T-cell surface. Molecular dissection
355 of gp41 demonstrated that the 3S peptide of gp41 is the active moiety. Plasma gp41 is most
356 likely a degradation product of circulating HIV or HIV-infected dead cells. We analyzed the
357 spatiotemporal mode of action of these two molecules when in the plasma. Our results suggest
358 that they act in two separate steps. Gp41 acts first and possibly modifies the CD4 membrane.
359 Gp41 may bind to CD4 T-cell membranes by binding to the gC1qR through its 3S sequence
360 (47, 48). This may change the membrane composition, possibly through induction of the fusion
361 of exocytic vesicles with the plasma membrane, as previously shown for NKp44L induction
362 (47). In the second step, these changes in lipid composition of the outer leaflet of the CD4 T-

363 cell may increase the binding and activity of PLA2G1B (49), making the modified membrane
364 a target of PLA2G1B.

365 PLA2G1B is the active moiety of the PLA2G1B/gp41 pair. At high concentrations, it appears
366 to act, in the absence of gp41, as a newly discovered immune modulator that acts on CD4 T
367 cells. Its unique properties, under these conditions, were defined in vitro or in vivo in mouse
368 experiments. PLA2G1B induces numerous aMMDs, trapping and inactivating the function of
369 receptors (γ c family, TCR,...), thus decreasing physiological activation, proliferation, and
370 survival. The results obtained with IFN α show that PLA2G1B works selectively on systems
371 that use pMMDs for physiological signaling. It is possible that, by digesting phospholipids,
372 PLA2G1B modifies membrane composition and changes its fluidity, allowing pMMDs to fuse
373 and form receptor-inactivating aMMDs.

374 Despite this broad activity, the action of PLA2G1B remains specific and does not act directly
375 on CD8 T cells. However, this mechanism may indirectly affect CD8 responses in HIV-infected
376 patients, which are mostly highly CD4 T cell-dependent (50). It will be of interest to extend our
377 studies to other immune cells, such as NK cells, which are highly dependent on the γ c cytokine
378 IL-15 for their activity (51).

379 In this context, we sought the origins of active PLA2G1B in the blood. We found the pancreas
380 to be a major source of PLA2G1B, followed by the duodenum, jejunum, and ileum
381 (Supplemental Figure 8, A-C). We used two different mAbs, 1C11 recognizing both
382 proPLA2G1B and active PLA2G1B and 14G9 specific for the active form, and observed that
383 proPLA2G1B is expressed in the endocrine pancreas while active PLA2G1B is mainly
384 expressed in the exocrine pancreas and intestinal tissues. We also observed low amounts of
385 *pla2g1b* transcripts in lymphoid cells (CD4 or CD8 T cells and Natural Killer cells) by qPCR,
386 whereas they were almost undetectable in myeloid cells (lung macrophages, mDC and pDC

387 dendritic cells) (Supplemental Figure 8D). The mechanism leading to the presence of active
388 and proPLA2G1B in the blood is still unknown. They may leak from the intestinal tract, where
389 proPLA2G1B is probably cleaved to form active PLA2G1B by proteolytic enzymes.
390 Conversion of proPLA2G1B into the active molecule may also take place at immune sites
391 where inflammatory cells produce proteolytic enzymes. Initially PLA2G1B was thought to play
392 solely a digestive role (35). Because of its function as an immunomodulator, demonstrated here,
393 it may also play a crucial role in the regulation of the lymphoid compartment of the gut immune
394 system. It may be involved in tolerance towards food and microbiota-derived components (52).
395 There is a high level of *pla2g1b* RNA in duodenum (Supplemental Figure 8A) and a high
396 concentration of active PLA2G1B protein in the intestinal lumen (Supplemental Figure 8B).
397 Thus, it may act directly on CD4 T cells or, alternatively, cofactors derived from bacteria or
398 viruses of the microbiota may be involved. These hypotheses may open new challenging areas
399 of investigation.

400 Our results also have other consequences for our understanding of the immunodeficiency of
401 HIV-infected patients. They show PLA2G1B to be the active component of the
402 PLA2G1B/gp41 pair and to contribute to the processes that renders most of the major
403 conventional CD4 T-cell subpopulations anergic. First, we have clearly demonstrated
404 dysfunction of the IL-7R/IL-7 induced microtubule and microfilament reorganization, therefore
405 blocking pSTAT5 NT. Furthermore, all γ c cytokines lost their function because of sequestering
406 of the γ c chains in macro-MMDs (aMMDs). This was verified by studying the blockade of IL-
407 2- and IL-4-induced pSTAT NT after PLA2G1B treatment. Furthermore, the TCR responses
408 induced by anti-CD3/anti-CD28 were also inhibited after PLA2G1B treatment. Inhibition of
409 the function of γ c cytokines and TCR responses leads to the blockade of antigen-specific
410 responses. Overall, our results show that Bumpy T cells obtained in vitro are anergic and
411 strongly suggest that the same is true for Bumpy T cells recovered from HIV-infected patients.

412 Furthermore, our data suggest that PLA2G1B is also involved in the CD4 lymphopenia
413 observed in HIV-infected patients. IL-7 is the main cytokine that controls homeostasis of the
414 CD4 compartment (53, 54). Thus, the PLA2G1B-induced defect of the IL-7R signaling
415 mechanism described here should significantly contribute to CD4 lymphopenia. Furthermore,
416 the number of surviving CD4 lymphocytes decreased after exposure to PLA2G1B in culture
417 (Figure 6, A and B). During these in vitro studies, we found numerous Annexin V-negative
418 Zombie-positive CD4 T-cells, which can be considered to be the direct consequence of
419 PLA2G1B activity, as they indicate the degradation of phosphatidylserine at the surface of the
420 dying cells (Figure 6, C-E). The activity of PLA2G1B on the membrane of dying cells led to
421 hypothesize an additional role of PLA2G1B in the removal of damaged cells, as previously
422 described for sPLA2 (35, 49). This may also contribute to the decreased in CD4 T-cell number.
423 In the future, Annexin V-negative dying cells could be used as a signature to study the effects
424 of PLA2G1B on the CD4 T cells of HIV-infected patients. This could be used to verify the
425 activity of PLA2G1B in vivo and to follow its neutralization after anti-PLA2G1B
426 immunotherapy.

427 PLA2G1B may represent a compelling therapeutic target for boosting immune responses in
428 people contaminated by HIV. In this context, the potential of the specific mAb 14G9 has to be
429 considered. It completely neutralized the effects of PLA2G1B in vitro, as measured by the
430 inhibition of pSTAT5 NT. This property was also verified in vivo, using a mouse model.
431 Furthermore, 14G9 accelerated the reversion of Bumpy cells in vitro, as measured by their
432 capacity to recover an IL-7 response. It also significantly reduced (up to >50%) the capacity of
433 PLA2G1B to decrease cell survival in vitro. Thus, neutralization of the deleterious effects of
434 the PLA2G1B/gp41 pair may be considered as a new therapeutic tool. It should be able to boost
435 the immune system early in infection, at a time when PLA2G1B is pathogenic as a consequence
436 of its synergy with gp41. Treatment during the beginning of ARV therapy, at a stage when the

437 viral load remains detectable, may increase the CD4 T cell-dependent immune defense and
438 improve the control of HIV infection. In addition, anti-PLA2G1B therapy could have positive
439 effects in patients infected by ARV-resistant HIV strains. After humanization, 14G9 could be
440 a drug candidate that could be used to boost the functions of the CD4 compartment and the
441 CD4-dependent immune responses of HIV-infected patients. By restoring IL-7 responses,
442 decreasing anergy, and contributing to an increase in CD4 counts, neutralization of PLA2G1B
443 may be one of the critical parameters towards remission of HIV infection (55–57).

444 The *in vivo* relevance of our data needs to be further underscored. The most significant data
445 comes from the analysis of the role of plasma from VPs. PLA2G1B in VP plasma is at
446 physiological concentrations and, in the presence of gp41, induces unresponsiveness of CD4
447 lymphocytes. The activity of plasma from VPs is well established. At the morphological level,
448 Bumpy T cells directly purified from the blood of VPs are indistinguishable from *in vitro* VP
449 plasma-induced Bumpy T cells. At the functional level, we found that aMMDs characterized
450 from purified CD4 T cells trap all γ c chains and subsequently showed that HD CD4 T cells
451 treated with VP plasma become unresponsiveness to γ c cytokines, such as IL-2, IL-4, and IL-7
452 (Figure 5, A and B). Inhibition or depletion of either PLA2G1B or gp41 abolishes the activity
453 of VP plasma (Figure 4, H and I and Figure 8, L and M). We thus propose a mechanism whereby
454 PLA2G1B is the active moiety and gp41 a cofactor or driver that targets PLA2G1B to the
455 surface of CD4 T cells. The activity of the VP plasma results from synergy between the two
456 molecules.

457 We then analyzed PLA2G1B and gp41 separately *in vitro* to understand the respective roles of
458 the two molecules. At high concentrations, PLA2G1B is active alone. High concentrations
459 appear to compensate for the absence of gp41. This allows characterization of the biochemical
460 and immunological properties of the active molecule. More significantly, at low concentrations,

461 cloned PLA2G1B was not active and did not induce unresponsiveness of the CD4 lymphocytes
462 in the absence of gp41. Under these experimental conditions, we clearly show that gp41 boosts
463 PLA2G1B activity (Figure 8, H-K). PLA2G1B becomes pathogenic only after CD4
464 lymphocytes have interacted with the gp41/3S peptide (Figure 8). Therefore, this in vitro
465 analysis further supports the model that synergy with viral gp41 is required to observe the
466 effects of low concentrations of PLA2G1B.

467 In addition, studies of three clinical settings confirm the relevance of our observations.
468 PLA2G1B activity strictly correlated with the presence of HIV particles. We only found
469 PLA2G1B activity in VPs who had HIV particles in the circulating blood and therefore gp41
470 in the plasma. In contrast, HIV controllers and patients treated for more than 10 years with
471 ARV, with an undetectable viral load in their plasma, had no detectable PLA2G1B activity.
472 Furthermore, we observed a negative correlation between the ability of plasma to induce
473 aMMDs in vitro and the CD4 counts of the patient source of plasma in a preliminary analysis
474 (Supplemental Figure 9A). Similarly, plasma from patients with CD4 counts below 300/mm³
475 more strongly inhibited pSTAT5 NT than plasma from patients with CD4 counts above
476 300/mm³ (Supplemental Figure 9B). Overall, these results corroborate the notion of a viral
477 cofactor/driver that synergizes with PLA2G1B to induce CD4 T lymphocyte unresponsiveness
478 in vivo.

479 It is noteworthy that the PLA2G1B/gp41 pair appears to be a new mechanism of
480 immunopathology, in which a physiological enzyme becomes pathogenic in the presence of
481 molecules derived from the pathogen. This system may play a role in diseases in which
482 immunodeficiency contributes to the emergence or progression of the disease. Preliminary data
483 obtained with hepatitis C, *Staphylococcus aureus*, and *Porphyromonas gingivalis* support this
484 concept.

485 **METHODS**

486 *Characterization of membrane microdomains (MMDs) in primary human CD4 and CD8 T*
487 *cells.*

488 Cell preparation and labelling of specific proteins were performed as previously described (34).
489 Briefly, purified cells were equilibrated in RPMI with 5% FBS for 2 h at 37°C and 5% CO₂
490 before plating them onto poly-L-lysine-coated coverslips for 20 min at 37°C. Cells were treated
491 with IL-7 (2 nM, 15 min, 37°C) then fixed with 1.5% paraformaldehyde (PFA, Electron
492 Microscopy Sciences) and rehydrated for 15 min in PBS/5% FBS. GM1 gangliosides were
493 labelled with AlexaFluor-coupled cholera toxin subunit B (CtxB-AlexaFluor488, C22841;
494 CtxB-AlexaFluor633, C34778; or CTxB-Biotin, C34779 and Streptavidin-AlexaFluor647,
495 S32357, Life Technologies).

496 MMDs were analyzed at the surface of fixed CD4 T cells and CD8 T cells from viremic patients
497 (VPc) and healthy donors (HDc) in response to IL-7 stimulation or not (Figure 1, A-C and
498 Supplemental Figure 4) or purified HD CD4 or CD8 T cells upon treatment with plasma
499 samples (from HD, VP, ART, or HIC), WT or H48Q PLA2G1B, PLA2GIIA, PLA2GIID, or
500 PLA2GX recombinant proteins for 30 min and stimulation with the cytokine for 15 min
501 (Figures 3 to 5).

502 Images were acquired below the diffraction limit using a Leica TCS STED-CW (31) (Figures
503 1 and 3 and Supplemental Figure 1) or Leica TCS SP8 STED 3X (Figure 4B) or above the
504 diffraction limit using an inverted laser scanning confocal microscope (LSM700, Zeiss or
505 LSM780 ELYRA PS.1, Zeiss) as previously described (34). Deconvolution was performed
506 using Huygens Pro software (Scientific Volume Imaging, Hilversum, The Netherlands). For
507 each condition, the top half of a representative CD4 T cell is shown from Z-stack images.
508 MMDs were counted on the entire surface of the purified CD4 T cells; an average of 50 cells

509 were examined for HD and between 15 to 50 for VP. We determined the number of MMDs in
510 Figures 1 and 3 or the percentage of cells with aMMDs on their surface in Figures 4 and 5.

511

512 *Phosphorylation and nuclear translocation of STAT (pSTAT NT).*

513 STAT phosphorylation and nuclear translocation in VP and HD CD4 T cells were analyzed by
514 microscopy after IL-7 stimulation (2 nM), or in HD CD4 and CD8 T cells incubated with
515 plasma samples from HD, VP, ART, or HIC (30 min), WT or H48Q PLA2G1B, PLA2GIIA,
516 PLA2GIID, or PLA2GX recombinant proteins, with or without neutralizing antibodies (30 min)
517 before a 15 min stimulation with 2 nM IL-7, IL-2, or IL-4 or 1 nM IFN- α 2. WT and H48Q
518 porcine PLA2G1B were used for the experiments shown in Figure 4D and F. The effect of the
519 anti-PLA2G1B 14G9 mAb on the recovery of a functional pSTAT5 NT response was studied
520 by pretreating CD4 T cells for 1 h with 250 nM PLA2G1B before the addition of 667 nM anti-
521 PLA2G1B mAb (14G9). All pre-treatments and stimulations were performed at 37°C.
522 Stimulation was stopped by addition of 4% PFA and incubation for 15 min at 37°C. Cells were
523 then permeabilized overnight at -20°C in a 90% methanol/water solution.

524 CD4 and CD8 T cells were stained using respectively anti-human CD4 (mouse anti- CD4 clone
525 RPA-T4, 555344, BD Biosciences; or goat anti-CD4, AF-379-NA, R&D/Novus) and anti-
526 human CD8 (mouse anti-CD8 clone RPA-T8, 555364, BD Biosciences), labelled with donkey
527 anti-mouse-AlexaFluor488 (A21202, Thermofisher) or donkey anti-goat-AlexaFluor488
528 (A11055, Thermofisher). Phosphorylation of STAT5 in response to IL-2 or IL-7 stimulation
529 was then revealed by staining with rabbit anti-pSTAT5 (9356, Cell Signaling Technology)
530 labelled with goat anti-rabbit-Atto 647N (15068; Active Motif) or donkey anti-rabbit
531 AlexaFluor555 (A31572, Life Technologies), that of STAT6 in response to IL-4 stimulation by
532 rabbit anti-pSTAT6 (9361, Cell Signaling Technology) labelled with anti-rabbit-
533 AlexaFluor488 (A11034 or A21206, Life Technologies), and that of STAT1 in response to

534 IFN- α 2 stimulation by rabbit anti-pSTAT1 (9167, Cell Signaling Technology) labelled with
535 anti-rabbit-AlexaFluor 488 (A11034 or A21206, Life Technologies).

536 Images were acquired below the diffraction limit with a DM16000CS/SP5 inverted laser
537 scanning confocal microscope using pulsed excitation STED (TCS STED, Leica) (58) or above
538 the diffraction limit using an inverted laser scanning confocal microscope (LSM700 or LSM780
539 ELYRA PS.1, Zeiss) as previously described (34). Deconvolution was performed using
540 Huygens Pro software (Scientific Volume Imaging, Hilversum, The Netherlands). The
541 appearance of pSTAT was measured using ImageJ software. pSTAT5 was quantified in the
542 cytoplasm and nucleus of the cells (Figures 1 and 3 and Supplemental Figure 1) where
543 indicated. The number of cells positive for nuclear pSTAT among > 200 in response to
544 cytokines was analyzed by confocal microscopy in Figures 4, 5, 7, and 8 and Supplemental
545 Figure 4.

546 *Study of the effect of PLA2G1B on human CD4 T-cell survival*

547 Purified CD4 T cells were cultured (7×10^6 cells/mL) in RPMI 1640 medium supplemented with
548 5% FBS (Life Sciences – Gibco). FBS was initially selected for its capacity to support efficient
549 CD4 T-cell activation in response to anti-CD3/CD28 stimulation, as measured by CD69 cell-
550 surface expression. The same FBS was also later found to support long-term survival of these
551 lymphocytes. CD4 T cells were treated with PBS, PLA2G1B, or the inactive mutant H48Q
552 PLA2G1B alone or with the anti-PLA2G1B mAb 14G9 (Figure 6 and Supplemental Figure 5)
553 or control isotype (Mouse IgG1, 16-4714-85, Thermofisher, Figure 6 and Supplemental Figure
554 5). The effect of PLA2G1B on CD4 T-cell survival was evaluated by a Moxy Z Mini Automated
555 Cell Counter (Moxy Z, Orflo technologies). Moxy Z measures cell counts, cell size, and cell
556 health. Cell health is evaluated via the Moxy Viability Index (MVI) value (59). The results based
557 on the MVI were analogous to those obtained by hemocytometer count of cells stained with

558 Trypan blue (0.1%).

559 For the Annexin V experiments, CD4 T cells were stained with AlexaFluor488-labelled
560 antibodies against CD4 (300519, Biolegend) for 30 min at 4°C, the Zombie Violet Fixable
561 Viability Kit (0.5 µL/test) (423114, Biolegend) and Annexin V-APC (5µL/test) (640941,
562 Biolegend) for 15 min at RT. Cells were analyzed with a cytoflex cytometer (Beckman Coulter)
563 and FlowJo software, version 10 (Tree Star).

564

565 *Determination of gp41 and 3S plasma cofactor peptide activity on healthy donor CD4 T cells.*

566 The effect of gp41 on PLA2G1B activity on CD4 T cells was assessed by incubating purified
567 CD4 T cells in PBS/1% BSA containing peptides, recombinant proteins, VP or HD plasma, or
568 the 10-30 kDa fraction previously depleted, or not, of PLA2G1B or gp41 (Supplemental
569 Material), together with recombinant PLA2G1B.

570 The binding of the viremic plasma cofactor to CD4 T cells was tested by first incubating the
571 PLA2G1B-depleted plasma with CD4 T cells for 15 min. Then, the adsorbed plasma was
572 collected and incubated with other CD4 T cells from the same donor for 30 min, alone or
573 together with PLA2G1B (Figure 8F).

574 The pretreatment effect of viremic plasma cofactor on CD4 T cells was tested by first incubating
575 the PLA2G1B-depleted plasma with CD4 T cells bound onto poly-L-lysine-coated coverslips
576 for 15 min. Then the supernatant was removed and the cells were washed and incubated for 30
577 min, with or without PLA2G1B (Figure 8G). pSTAT5 NT was analyzed in CD4 T cells
578 incubated with the adsorbed supernatants.

579 The effect of the recombinant gp41 and 3S gp41 peptide on PLA2G1B activity was tested by
580 pretreating the cell suspension for 15 min with 40 µl of the recombinant gp41 protein, peptides,
581 with subsequent addition of 10 µl PLA2G1B for 30 min (Figure 8, H-K). The regulation of

582 PLA2G1B by endogenous gp41 was tested by treating the cell suspension for 30 min with 50
583 μ l of plasma dilutions or 10-30 kDa plasma fraction, previously depleted or not of gp41
584 (Figures 8, L and M). pSTAT5 NT inhibition was examined by microscopy as described above.

585

586 Additional reagents and procedures are detailed in the online Supplemental Material, which
587 includes information on study design and human sample collection, recombinant proteins and
588 peptides, cell purification and culture, detergent-resistant microdomain (DRMs) analysis,
589 western-blot analyses, analysis of the IL-7 receptor (IL-7R) diffusion rate at the surface of
590 living CD4 T lymphocytes, the study of the effect of cytoskeleton inhibitors on pSTAT5 NT,
591 analysis of cytoskeleton organization, identification of PLA2G1B as the active component in
592 VP plasma, active human PLA2G1B structure determination, lipidomic analysis, ELISA,
593 quantitative real-time PCR, immunodepletion experiments, flow cytometry analyses, *in vitro*
594 experiments on mouse T cells, and *in vivo* experiments in mice.

595

596 *Statistics*

597 Statistical parameters, including the exact value of n, precise measures (mean \pm SD in all
598 Figures, with the exception of the mean \pm SEM in Figure 7, B-G), statistical significance, and
599 tests used for each analysis are reported in the figures and figure legends. Analyses were
600 performed using GraphPad Prism (GraphPad Software Inc.).

601 For experiments on human cells, one donor represents one experiment. For experiments on
602 mice, the number of pooled mice from n independent experiments is shown.

603 Correlations between two variables were evaluated by Pearson's correlation and linear
604 regression.

605 Data were analyzed using the two-tailed unpaired *t*-test for two groups or ANOVA with
606 correction for multiple comparisons (Tukey's, Dunnett's, or Sidak's) when the distribution was
607 gaussian according to the D'Agostino & Pearson omnibus test. The effect of PLA2G1B on the
608 survival of mouse CD4 T cells in G0 to G5 was analyzed by two-way ANOVA with Dunnett's
609 correction for multiple comparisons using the control condition as the control group. The anti-
610 PLA2G1B effect on CD25 expression and the survival of PLA2G1B-treated mouse CD4 T
611 cells, as well as the kinetics of the effect of PLA2G1B injection on the percentage of cells
612 showing pSTAT5-NT, were analyzed by two-way ANOVA with Sidak's correction for
613 multiple comparisons. When data were not Gaussian, Mann-Whitney's non parametric test was
614 used to compare two groups and Kruskal-Wallis test was used when more than two groups were
615 compared. When Kruskal-Wallis test was significant, two-by-two comparisons were conducted
616 to identify groups which differed, but applying a Bonferroni correction. The level of
617 significance is indicated as **p* < 0.05, ***p* < 0.01, and ****p* < 0.001 in all figures.

618 *Study approval*

619 *Human study:* This study was supported by the ANRS and approved by the Comité des
620 Personnes Ile-de-France VII under number 05-15. All participants were adults and provided
621 written informed consent prior to inclusion in the study.

622 *Animal studies:* All animal experiments described in the present study were conducted at the
623 Institut Pasteur according to European Union guidelines for the handling of laboratory animals
624 (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm) and were approved by
625 the Institut Pasteur Animal Care and Use Committee (CETEA 89, Institut Pasteur de Paris) and
626 the Direction Sanitaire et Vétérinaire de Paris under permit number 2016-0004 and
627 APAFIS#6453-2016071912038344 v2. All experiments were subject to the three R's of animal
628 welfare (refine, reduce, and replace).

629 **AUTHOR CONTRIBUTIONS**

630 J.P., T.R., and F.B. designed and conducted the experiments on human and mouse cells. The
631 study was initiated by T.R and continued by J.P, who participated in the writing of the
632 manuscript. D.G. performed the TCS SPS STED analysis. T.R. designed and conducted the
633 plasma chromatography, biochemical analysis, microscopy image analysis, MS, and
634 bioinformatics data analysis. L.J. participated in the mAb characterization, ELISA
635 development, and recombinant protein production. A.M. participated in the characterization of
636 anti-gp41 mAb. A.H. and F.S. designed and conducted the structural analysis. J.A. and E.R.-
637 M. participated in the studies of plasma from HIV-infected patients. L.T. provided PLA2G1B
638 protein. G.L. contributed to the design of certain experiments, provided ideas and models, and
639 shared resources. J.T. was the lead senior author for this paper.

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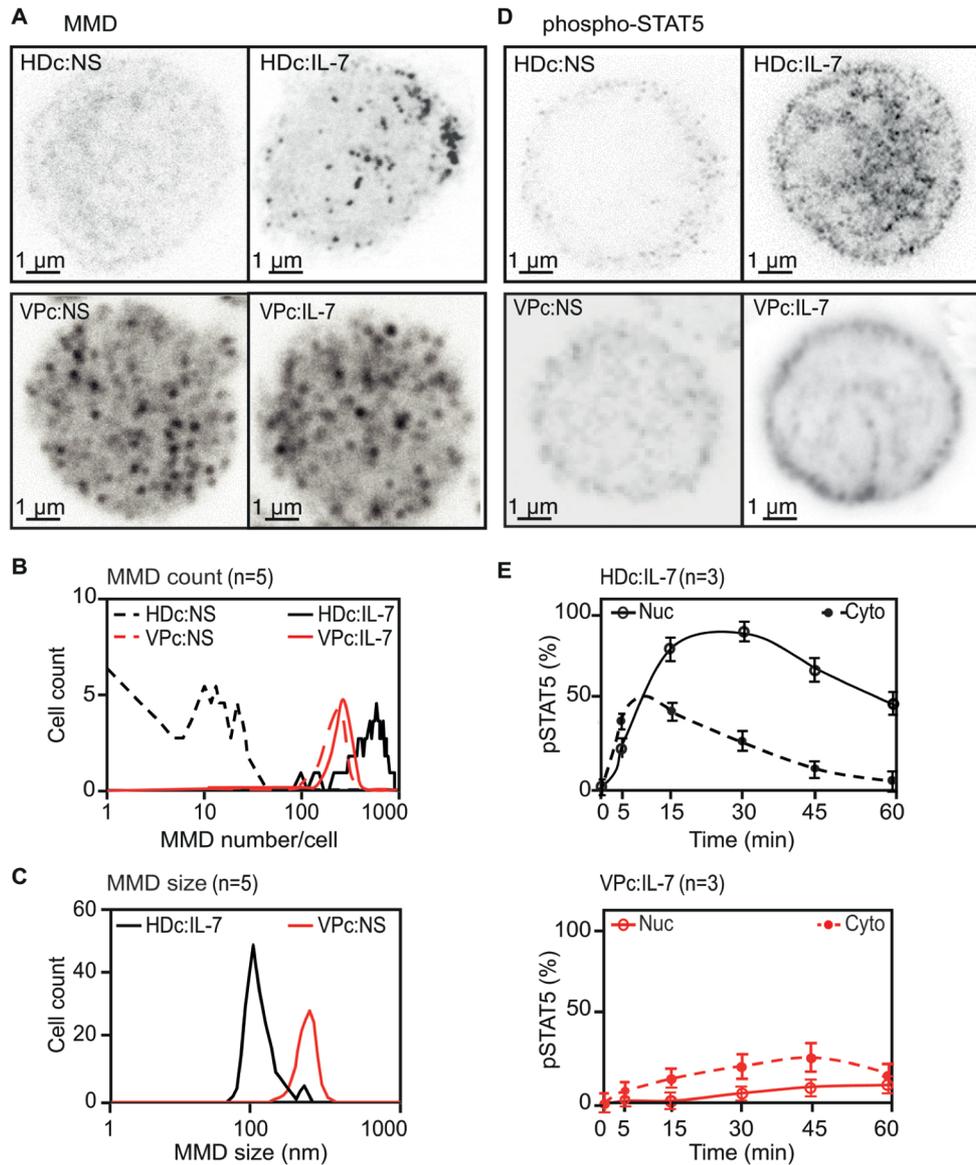


Figure 1. Characterization of Bumpy T cells from HIV-infected patients.

(A) MMD analysis by CW-STED microscopy. From top to bottom, purified HD CD4 T cells (HDc) and VP CD4 T cells (VPc). For each group, the top half of a representative non-stimulated (NS) CD4 T cell, or after IL-7 stimulation, is shown from Z-stack images. (B) Quantification of MMDs on the surface of HD CD4 cells (HDc) and VP CD4 cells (VPc) before (NS) and after IL-7 stimulation. (C) Size of MMDs at the surface of IL-7-stimulated HD cells (HDc:IL-7) and VP cells before stimulation (VPc:NS). Lines represent the mean values. (D) Analysis of IL-7-induced phosphorylation and nuclear translocation of STAT5 by pulsed-STED microscopy (0.5 μ m slices) in non-stimulated and IL-7-stimulated HD CD4 T cells (top) or VP CD4 T cells (bottom). (A-D) An average of 50 cells from each HD and 15-50 cells from each VP (HIV RNA/ml = 49,144 \pm 33,689) were examined from five donors in each group and representative images are shown in A and D. (E) The kinetic of pSTAT5 in the nucleus (Nuc) and cytoplasm (Cyto) of HD and VP CD4 T cells after IL-7 stimulation was measured using ImageJ and represented as the mean \pm SD for three donors.

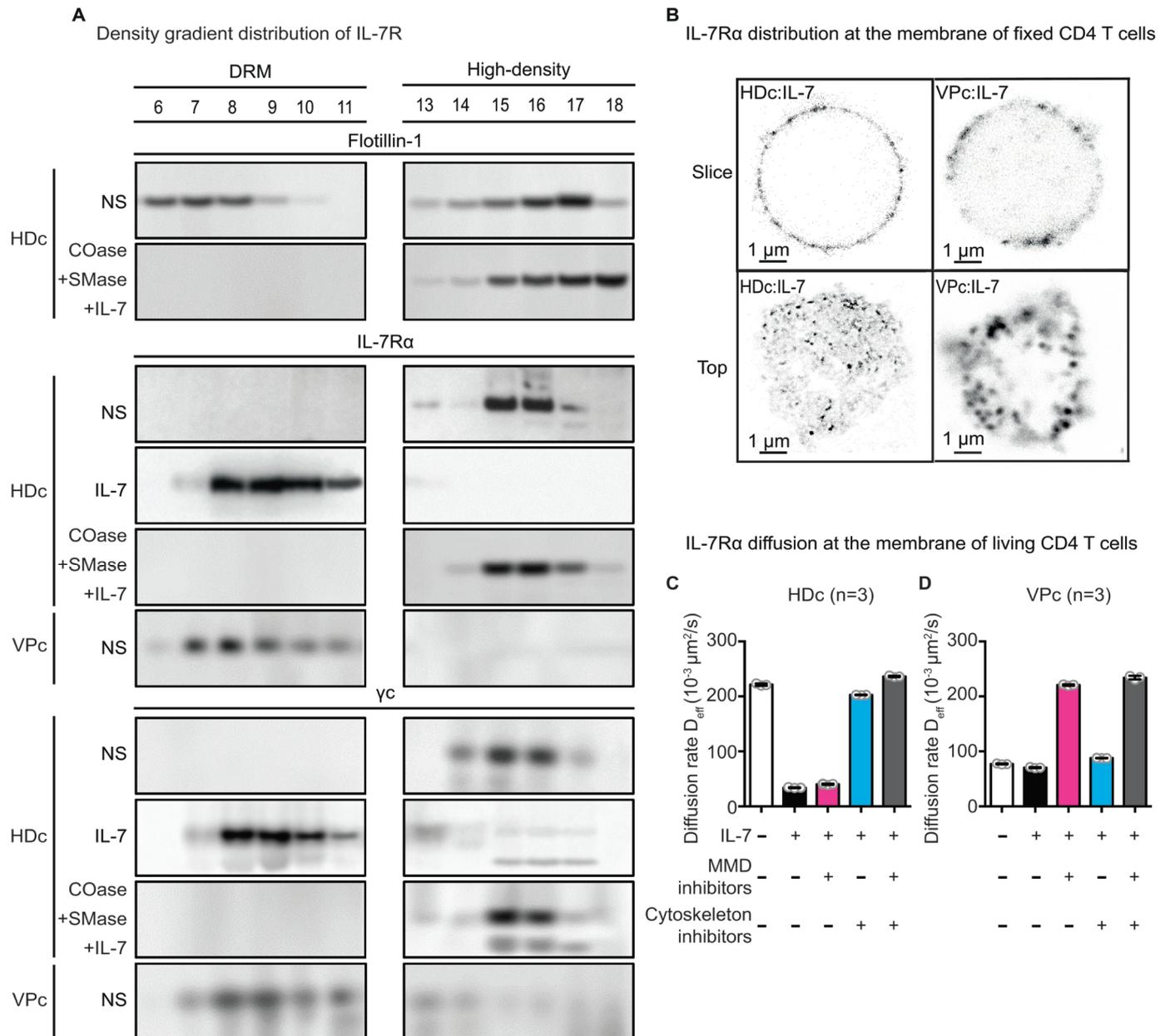


Figure 2. Analysis of membrane domains and IL-7R distribution on the surface of HD and VP CD4 T cells.

(A) Purified CD4 T lymphocytes were lysed (0.5% Triton X-100) and the lysates were loaded onto a 5-40% sucrose gradient. After 16 h of centrifugation (50k rpm) at 4°C, 18 fractions were collected (#1 left=tube top=5% sucrose; #18 right=tube bottom=40% sucrose). Each fraction was analyzed by SDS-PAGE (2 gels). Flotillin-1 was used as a marker to indicate low-density fractions corresponding to DRM. IL-7R α and γ chains were revealed by western blotting. Results are shown for purified non-stimulated HD CD4 T-cells (HDc:NS), IL-7-stimulated HD CD4 T cells (HDc:IL-7), or HD CD4 T-cells pre-treated with cholesterol oxidase (Coase: 31 μM , 25 min) and sphingomyelinase (SMase: 2.7 μM , 5 min), and IL-7-stimulated (HDc:Coase+SMase/IL-7), as well as non-stimulated VP CD4 T cells (VPc:NS) (n=3 donors). (B) IL-7R α chain localization at the membrane of CD4 T cells from HDs and VPs was analyzed by CW-STED. Images of a section (slice) and of the top view (top) of representative cells are shown among 50 cells per HD (3 donors) and 15 to 50 cells per VP CD4 T cells (3 donors). (C, D) The effect of cytoskeletal reorganization and MMD inhibitions on IL-7R compartmentalization was evaluated by measuring the two-dimensional effective diffusion rates (D_{eff}) of the IL-7R α chain by fluorescence correlation spectroscopy (FCS) as described in Tamarit et al., 2013. Histograms represent the mean \pm SD of the effective diffusion rate D_{eff} in each condition at the surface of (C) HD (3 donors) and (D) VP CD4 T cells (3 donors).

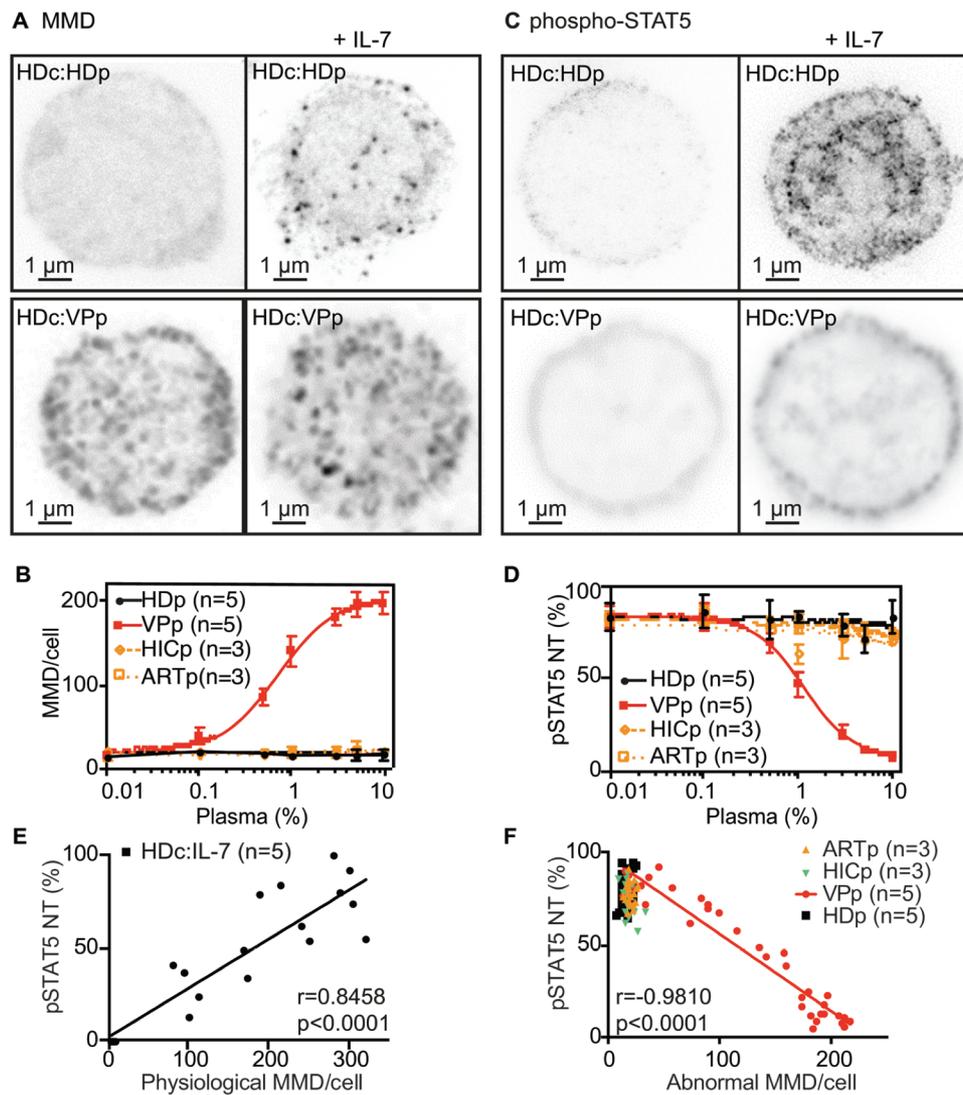


Figure 3. Induction of Bumpy CD4 T cells by plasma from HIV-infected patients. (A) CW-STED images of MMD on HD CD4 T cells treated with 10% HDp (HDc:HDp) or VPp (HDc:VPp) before and after IL-7 stimulation. (B) Dose-effect of plasmas from HD (HDp, n=5), VP (VPp, n=5, HIV RNA/ml = 49,144 \pm 33,689), HIV-controllers (HICp, n=3), and ART-treated donors (ARTp, n=3) on the number of MMDs per HD CD4 T cell. (C) Pulsed-STED images of pSTAT5 of HD CD4 T cells pre-treated with 10% plasma from HDs or VPs. (D) Plasma dose-effects on pSTAT5 NT in IL-7-treated HD CD4 T cells. Data are represented as the mean \pm SD. (A-D) For each condition, an average of 50 HD CD4 T cells were analyzed from five donors and representative images are shown in A and C. (E) Pearson's correlation between the kinetics of pSTAT5 NT and the number of physiological MMDs throughout IL-7 activation of HD cells (up to 60 min). Linear regression for the mean of the five HD plasma samples is shown. (F) Pearson's correlation between pSTAT5 NT and abnormal MMD per HD CD4 T cell treated with various amount of plasma from HD (HDp, n=5), VP (VPp, n=5, HIV RNA/ml = 49,144 \pm 33,689), HIV-controllers (HICp, n=3) and ART-treated donors (ARTp, n=3). Linear regression for the mean of the five VP plasma samples is shown.

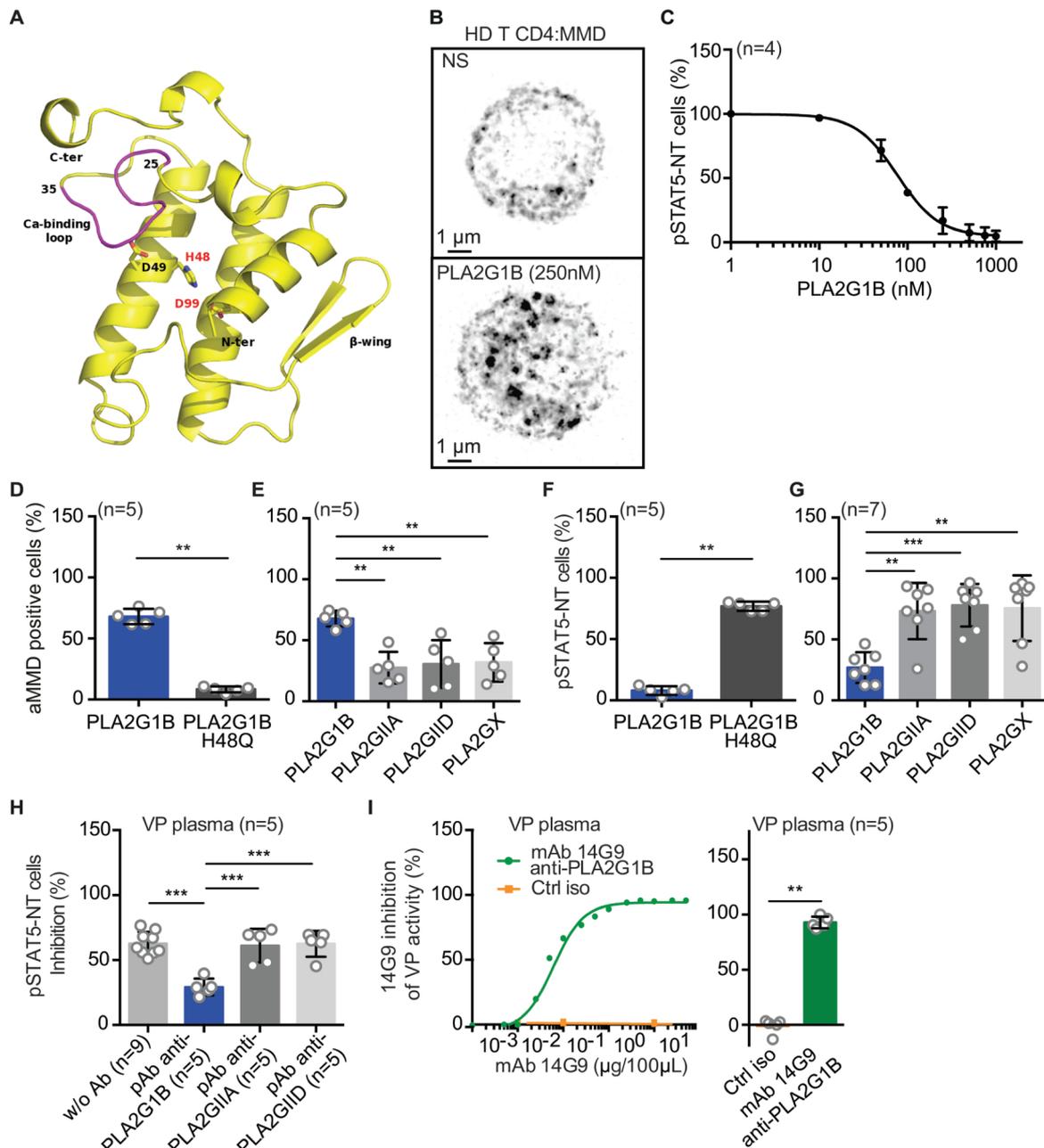


Figure 4. Cloned plasma PLA2G1B induces the Bumpy T-cell phenotype.

(A) Crystal structure of PLA2G1B. (B) PLA2G1B effect on MMD formation followed by STED (representative of two experiments at 250 nM and verified at 500 nM and 1 μ M). (C) Dose-effect of PLA2G1B on IL-7-induced pSTAT5 NT in HD purified CD4 T cells after analysis of confocal images. Results are shown as the mean \pm SD from four donors. (D-G) The effects on aMMD formation (D, E) and pSTAT5 NT (F, G) in CD4 T cells at 250 nM of wildtype PLA2G1B were compared to those of the non-active mutant H48Q (D, F) and of other PLA2s (PLA2GIIA, PLA2GIID, or PLA2GX) (E, G). Results are shown as the mean \pm SD from five (D-F) or seven donors (G). (H) VP plasma (3%, from 5 donors) was depleted with anti-PLA2G1B, anti-PLA2GIIA or anti-PLA2GIID rabbit polyclonal antibodies (100 μ g/mL). The effect of depletion was analyzed by following pSTAT5 NT in IL-7-stimulated-CD4 T cells (n=3 donors) incubated with depleted plasma. Results were normalized to the response obtained with HD plasma and are shown as the mean \pm SD. (I) Effect of VPp treated with various doses of neutralizing anti-PLA2G1B mAb 14G9 on pSTAT5 NT in CD4 T cells from one donor and the effect of 100 μ g/mL of 14G9 mAb on pSTAT5 NT in CD4 T cells from five donors. * p <0.05, ** p <0.01 and *** p <0.001 by the Mann-Whitney t -test (D, F, I) and by the Kruskal-Wallis followed by the Mann-Whitney test with p-values adjusted for multiple comparison between groups (E, G) or one way ANOVA (H) with Tukey's correction for multiple comparisons.

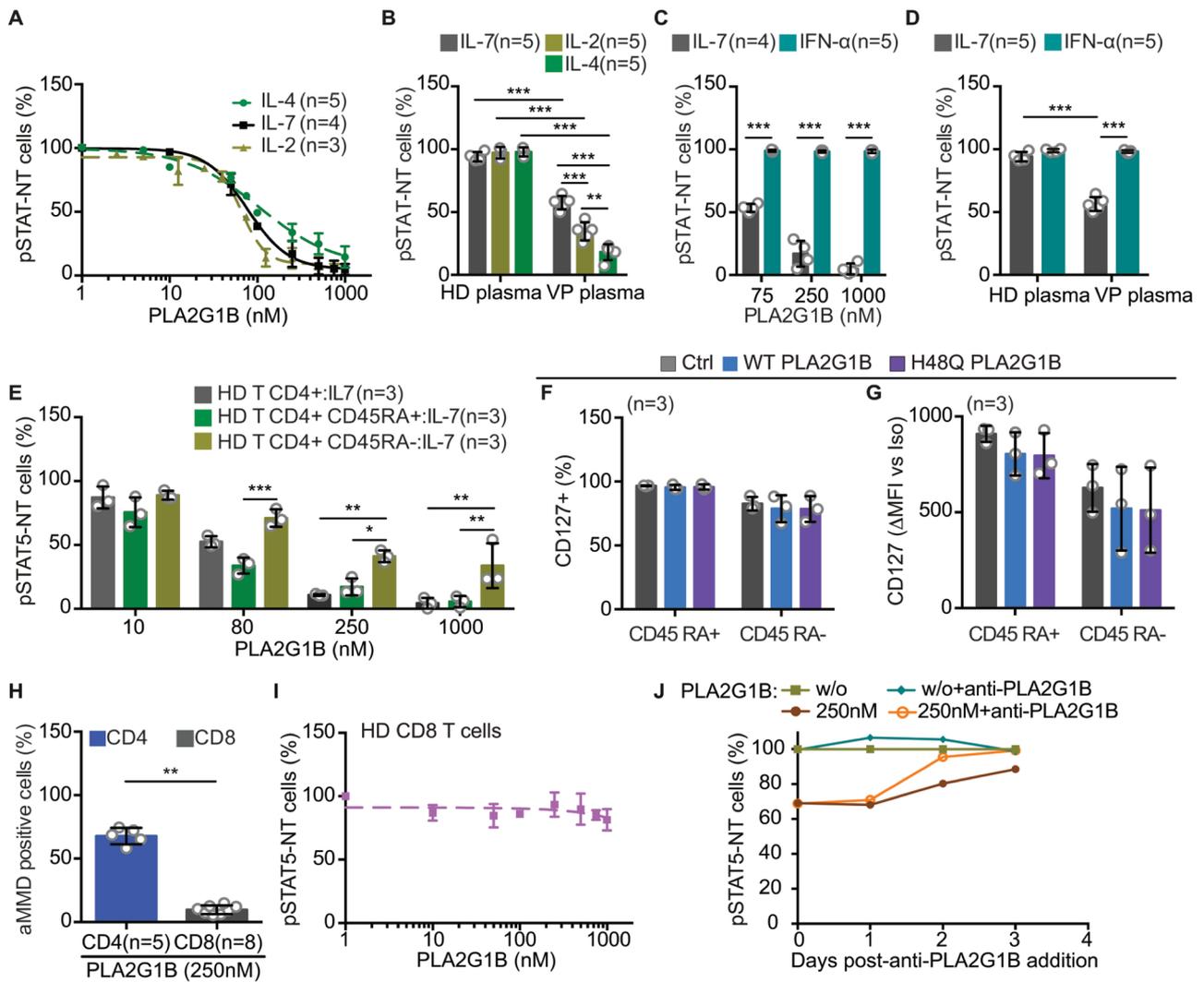


Figure 5. Effect of PLA2G1B on CD4 T-cell subpopulations, specificity, and reversion.

(A) Dose-effect of PLA2G1B (IL-7: n=4, IL-2: n=3 and IL-4: n=5) and (B) of 1% HDp (IL-7: n=4, IL-2 and IL-4: n=3) and VPp (n=5) on IL-2, IL-4, and IL-7-induced pSTAT translocation in CD4 T cells. (C) Effects of PLA2G1B (IL-7: n=4, IFN- α : n=5) and (D) plasmas (HD (n=4) or VP (n=5), 1%) on IL-7-induced pSTAT5 NT and IFN- α -induced pSTAT1 NT in CD4 T cells (n = 5 donors). (E) The effect of PLA2G1B (30 min) on IL-7-induced pSTAT5 NT was analyzed in total (HD T CD4+:IL-7), naïve (HD T CD4+ CD45RA+:IL-7), and memory (HD T CD4+ CD45RA-:IL-7) CD4 T cells from the same donor in response to IL-7 (n=3 donors). (F) Percentage of CD127+ cells among and (G) CD127 expression (delta anti-CD127 MFI minus isotype control MFI) on CD45RA+ and CD45RA- CD4 T cells after treatment with 1 μ M WT or H48Q PLA2G1B (see gating strategy in Supplemental Figure 3A, n=3 donors). (H) Effect of PLA2G1B (250 nM) on aMMD induction in CD4 T cells (n=5) and CD8 T cells (n=8) and (I) on IL-7-induced pSTAT5 NT in CD8 T cells (dose-effect, n=3). (A-I) Results are shown as the mean \pm SD. * p <0.05, ** p <0.01 and *** p <0.001 by one way ANOVA (B-D) and two way ANOVA (E) with Tukey's correction for multiple comparison or ** p <0.01 by the Mann-Whitney two tailed unpaired t -test (H). (J) Anti-PLA2G1B treatment accelerates the recovery of a functional pSTAT5 NT response of PLA2G1B-treated CD4 T cells to IL-7. The results of one representative of three experiments are presented.

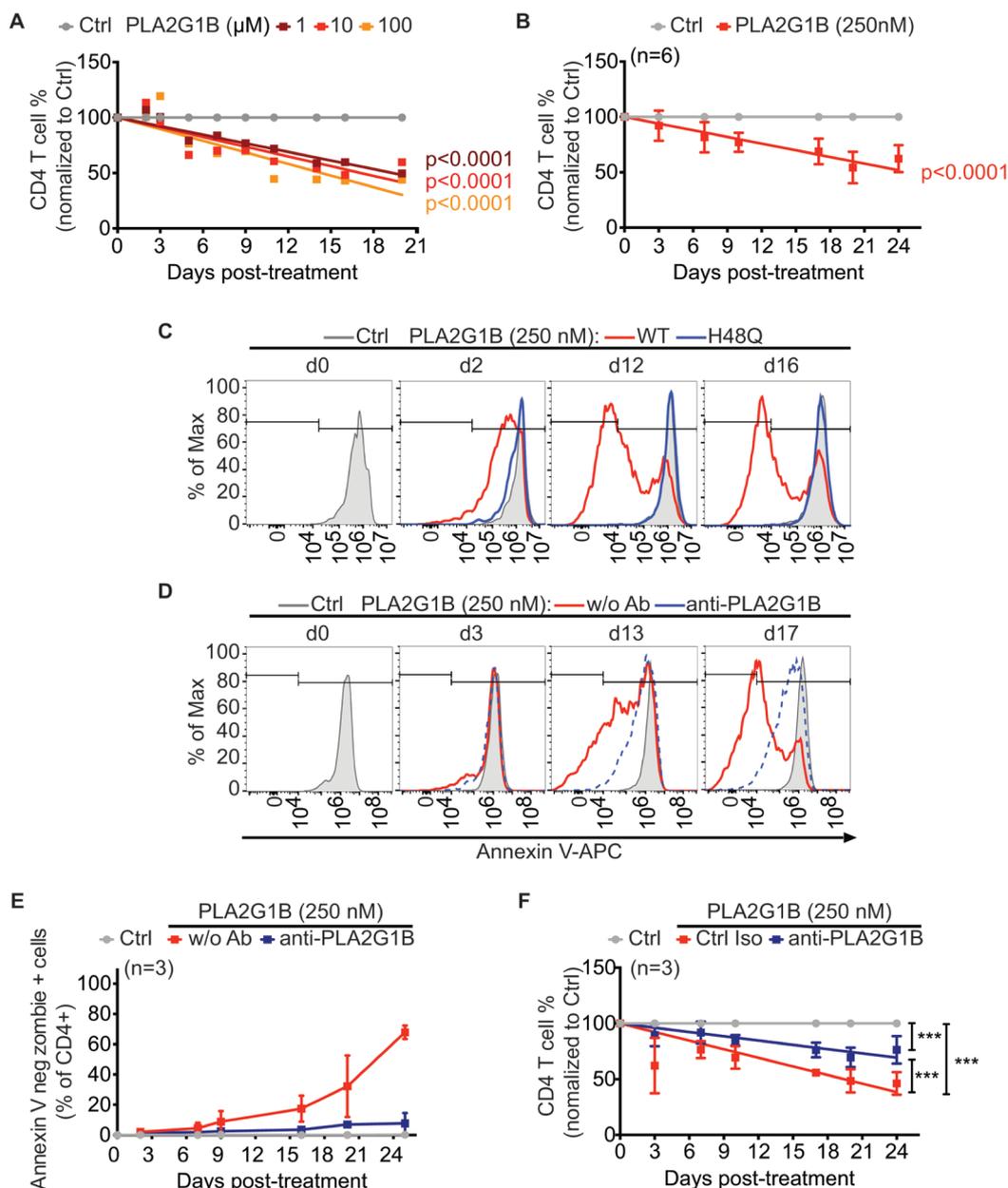


Figure 6. PLA2G1B acts on dying CD4 T cells and reduces CD4 T-cell survival.

(A, B) PLA2G1B reduces the survival of human CD4 T cells. (A) Cells were treated with PBS (Ctrl) or various amounts of PLA2G1B (1, 10, 100 μ M) for one experiment. Results are shown as the percentage of CD4 T-cell counts normalized to the number of Ctrl cells at each time point. (B) Cells were treated with PBS (Ctrl) or 250 nM PLA2G1B (n=6 donors). Results are shown as the mean \pm SD of the percentage of CD4 T-cell counts normalized to the number of Ctrl cells at each time point. (A, B) The lines show the linear regression and the p-values indicate the significance of difference with control. (C-E) PLA2G1B acts on dying CD4 T cells and digests phosphatidylserine. FACS analysis of CD4⁺ T cells for Annexin V-APC on Live/Dead Marker (Zombie-Violet) positive cells after treatment with (C) 250 nM PLA2G1B WT or H48Q or (D) 250 nM PLA2G1B with anti-PLA2G1B (14G9) or not (w/o Ab). (C, D) Annexin V-APC labelling (MFI) at various time points post-treatment are presented (one representative experiments of two in C and three in D is presented). (E) Results are shown as the mean \pm SD of the percentage of Annexin V-negative Zombie-positive CD4 T cells after treatment with PBS (Ctrl), PLA2G1B alone (w/o Ab), or with anti-PLA2G1B (14G9) (n=3 donors). (F) Anti-PLA2G1B treatment inhibits the effect of PLA2G1B on the survival of CD4 T cells. Results are shown as the mean \pm SD of the percentage of CD4 T-cell counts normalized to the number of Ctrl cells at each time point (n=3 donors). Lines show the linear regression and p-value indicate the significance difference between experimental conditions, ****p*<0.001.

Figure 7. Immunological effects of hPLA2G1B on mouse CD4 T cells in vitro and in vivo.

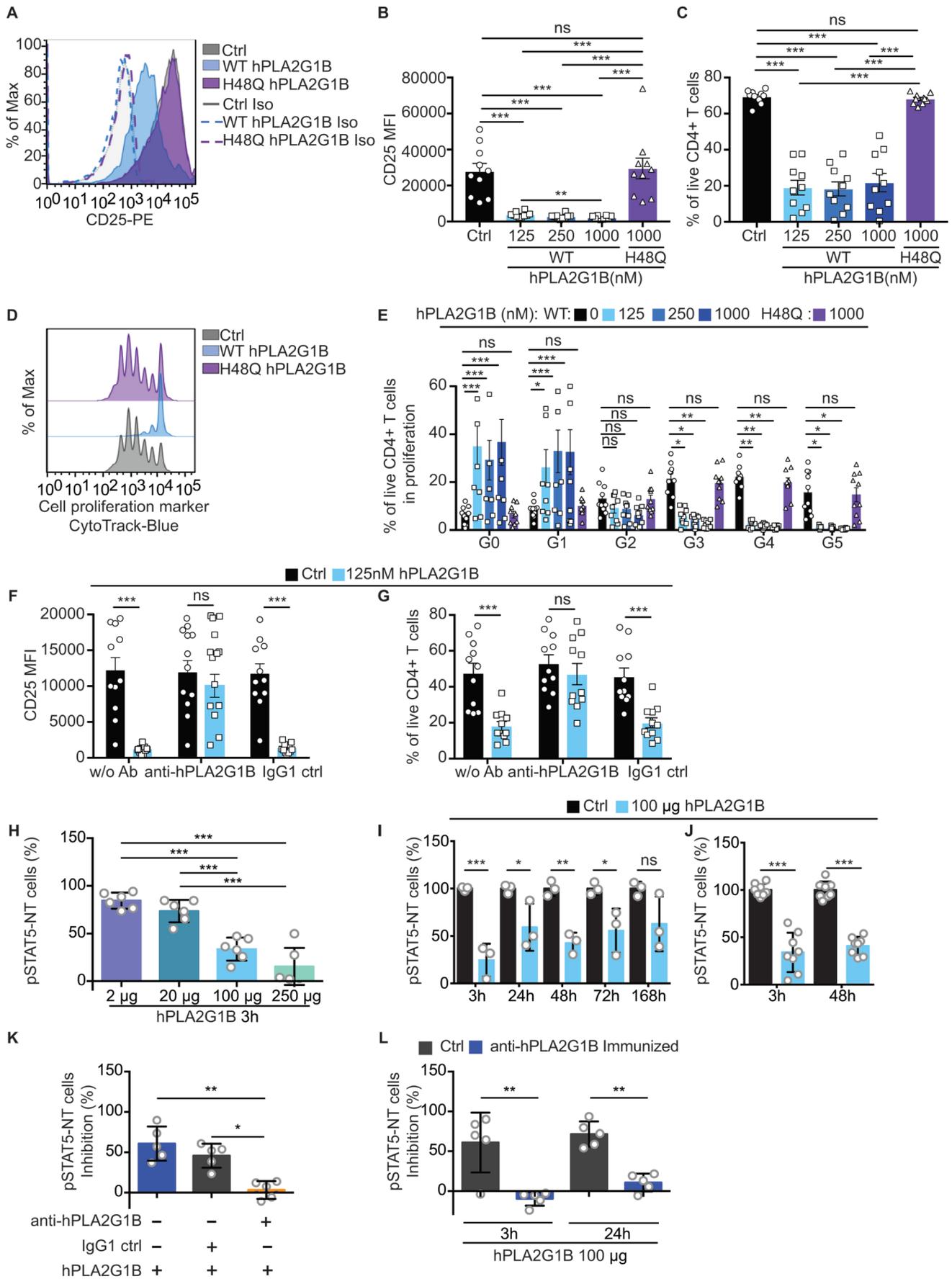


Figure 7. Immunological effects of hPLA2G1B on mouse CD4 T cells in vitro and in vivo. (A-G) FACS analysis of the effect of hPLA2G1B on mouse CD4⁺ T cells after anti-CD3/CD28 and IL-2 stimulation (five days, see gating strategy on Supplemental Figure 6C). (A-E) mCD4⁺ T cells were pretreated with WT or H48Q hPLA2G1B. (A) CD25 expression after treatment with 125 nM. (B) CD25 expression (MFI) and (C) cell survival (n=3, 10 mice). (D) mCD4⁺ T-cell proliferation profile after treatment with 125 nM. (E) Percentage of live mCD4⁺ T cells per cell generation (Go to G5; n=3, 9 mice). (F, G) Effects of mAb anti-PLA2G1B 14G9 in vitro treatment on 125nM hPLA2G1B action on CD4⁺ T-cell survival and CD25 expression (n=4, 11 mice). (H-L) In vivo effects of hPLA2G1B on CD4 T-cell response to IL-7. Spleen CD4 T cells were isolated after intraperitoneal injection into C57BL/6 mice and the ex vivo pSTAT5 NT response to IL-7 was evaluated by confocal microscopy with an average of 200 cells examined for each condition. Effect of hPLA2G1B injection at several doses of PLA2G1B for 3 h (6 mice/2 experiments, H) and at several times post-injection (3 mice/1 experiment, I; 8 mice/2 experiments, J). (K) Effects of mAb anti-hPLA2G1B 14G9 injected in vivo on the hPLA2G1B (100 µg, 3 h) response (5 mice/1 experiment). (L) Inhibition of the effects of hPLA2G1B after injection into hPLA2G1B/BSA-immunized mice (5 mice/1 experiment). Results are shown as the mean ± SEM (B, C and E-G) ± SD (H-L). **p*<0.05, ***p*<0.01 and ****p*<0.001 are adjusted *p*-value for multiple comparisons performed by Kruskal-Wallis test *p*<0.001, followed by the Mann-Whitney test (B) and two-way ANOVA with correction for multiple comparisons of Tukey (C, H, J-L), Dunnett, with the condition w/o PLA2G1B as a control group (E), or Sidak (F, G, I).

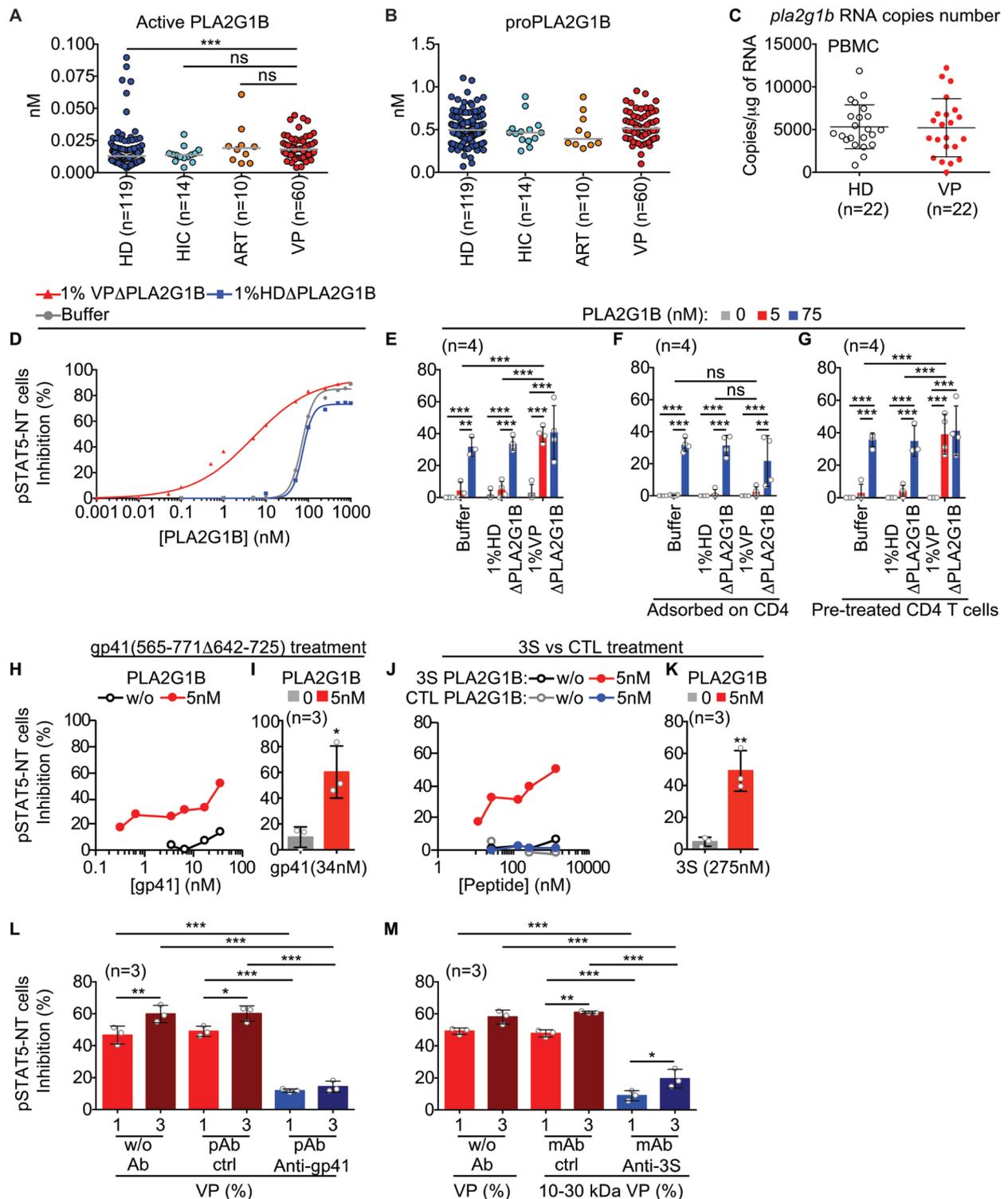


Figure 8. Synergy between PLA2G1B activity and gp41.

(A, B) ELISA quantification of PLA2G1B in plasma from HD, VP, HIV-controllers (HIC) and ART-treated donors (ART) (median is shown). The Kruskal-Wallis test p-value was 0.0025 on A and then multiple comparisons were performed using the Mann-Whitney test. *** $p < 0.001$. (C) Level of *pla2g1b* RNA in PBMCs from HDs and VPs. Results are shown as mean \pm SD of the number of copies of *pla2g1b*/μg of total RNA. (D) Inhibitory activity of PLA2G1B diluted in PBS buffer or plasma from HDs or VPs previously depleted (Δ) of endogenous PLA2G1B ($p < 0.0001$ non-linear regression in VPP relatively to HDp/buffer). (E) The same experiment as in (D) with 1% plasma and 5 or 75 nM PLA2G1B. (F) VP plasma previously adsorbed on CD4 T cells. Adsorbed plasma or buffer were collected and used to treat other CD4 T cells together with PLA2G1B or not. (G) PLA2G1B activity on VP plasma-pretreated CD4 T cells. CD4 T cells were pretreated with plasma or buffer, then plasma or buffer were removed and PLA2G1B was added, or not, to the pretreated CD4 T cells. (H, I) PLA2G1B inhibitory activity in the presence of the gp41 fragment (J, K) or 3S or control (CTL) peptides. (L, M) Inhibitory activity of 1% or 3% VP plasma depleted with anti-gp41 (gp41) polyclonal antibody (pAb) (L) or anti-3S gp41 monoclonal antibody (Anti-3S) (M), control (ctrl) or not depleted (w/o Ab) on CD4 T cells. D, H and J show one representative dose-response experiments among 2-3. (E-G, I, K-M) results are shown as the mean \pm SD of the percentage of pSTAT5 NT cells inhibition on 3-4 donors, as indicated. * $p < 0.05$, ** $p < 0.01$ by two tailed unpaired *t*-test (I and K), *** $p < 0.001$ by ANOVA with Tukey's correction for multiple comparisons (E-G, L and M).