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Abstract

The effect of anti-HIV-1 antibodies on complement activation at the surface of infected cells remains partly understood. Here, we show that a subset of anti-Envelope (Env) broadly neutralizing antibodies (bNAbs), targeting the CD4 binding site and the V3 loop, triggers C3 deposition and complement-dependent cytotoxicity (CDC) on Raji cells engineered to express high surface levels of HIV-1 Env. Primary CD4 T cells infected with laboratory-adapted or primary HIV-1 strains and treated with bNAbs are susceptible to C3 deposition but not to rapid CDC. The cellular protein CD59 and viral proteins Vpu and Nef protect infected cells from CDC mediated by bNAbs or by polyclonal IgGs from HIV-positive individuals. However, complement deposition accelerates the disappearance of infected cells within a few days of culture. Altogether, our results uncover the contribution of complement to the antiviral activity of anti-HIV-1 bNAbs.

Introduction

The complement is a network of proteins initially described for its ability to induce cell toxicity [1]. Due to its ancestral origin, the complement is highly intricated within innate and adaptive immune systems [2]. The three canonical mechanisms of complement activation, known as classical, alternative and lectin pathways, converge to the proteolytic cleavage of component 3 (C3) into C3a and C3b. The Fc regions of membrane-bound antibody multimers initiate the classical pathway [3]. Covalent binding of C3b on targeted membranes promotes different events. Cleaved forms of complement proteins are recognized by complement receptors (CRs), expressed by numerous immune cells, such as macrophages, NK cells, or dendritic cells (DC) [4]. CRs cooperate with Fc receptors (FcR) to modulate immune responses against antibody-decorated targets [5]. Complement deposition at the plasma membrane triggers intrinsic signaling pathways impacting cell fate or activation [6–8]. C3 deposition may lead to the assembly of the C5b-9 complex, also termed the membrane attack complex (MAC), that induces membrane disruption and cell death [9]. Intracellular sensing of complement C3 also activates cell autonomous immunity [10].

The HIV-1 glycoproteins gp120 and gp41 assemble as a trimer to form the viral envelope (Env). Recognition of the receptor CD4 reorganizes Env to allow fusion by exposing the fusion peptide of gp41. Env exists in at least two conformations, a native "close" configuration and a CD4-bound "open" conformation (reviewed in [11]). Numerous broadly neutralizing monoclonal antibodies (bNAbs) targeting the HIV-1 Env glycoprotein have been isolated [12,13]. These antibodies target the CD4 binding site (CD4bs), the *N*-glycans of V1/V2 and V3 loops, the gp41 membrane proximal external region (MPER), and the gp120/gp41 interface [12]. In animal models, infusion of bNAbs protects against HIV-1 acquisition, decreases viral load [14,15], modulates host immune responses [16,17], and, when associated to latency-reversal agents, delays viral rebound after treatment interruption [18,19]. At least nine

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bNAbs are currently under clinical evaluation, either alone or in combination, and display antiviral activity in humans [20].

The activity of bNAbs goes beyond neutralization. Their Fc region is required for optimal *in vivo* efficacy [21–23]. bNAbs kill HIV-1-infected cells by inducing antibody-dependent cellular cytotoxicity (ADCC) [24,25] and mediate antibody-dependent cellular phagocytosis [26]. A growing body of evidence highlights the importance of complement in antibody-based therapies [27]. However, little is known about the ability of bNAbs to activate the complement system, and how this activation may modulate their function.

HIV-1 virions are vulnerable to complement-mediated lysis (i.e., virolysis) [28,29]. Plasma from HIV-1-infected individuals mediates virolysis [30,31]. During primary infection, virolysis inversely correlates with viremia, suggesting that it may limit viral replication [30]. However, HIV-1 counteracts virolysis by incorporating host proteins CD55 and CD59 during budding [32,33]. CD55 prevents the formation of C4b2a and C3bBb C3-convertases, whereas CD59 interferes with MAC assembly. HIV-1 may also take advantage of the complement pathway [34]. C3 opsonization of virions favors the infection of CR-expressing cells and leads to HIV-1 persistence in germinal centers (GC) by trapping viral particles on follicular DC [34].

Upon infection, cell lines display variable susceptibility to complement-dependent cytotoxicity (CDC) mediated by anti-HIV antibodies [35–39]. This may be explained by the inconstant capacity of polyclonal IgGs to recognize cell-associated Env, and by various levels of CD46, CD55, and CD59 expressed in different cells [32,40,41]. Cells that have captured viral material [42,43], or at an early stage of infection, have been proposed to be the main target of CDC [35]. Anti-V1/V2 glycans [35] and anti-V3 antibodies [36,44] were suggested to be better CDC inducers than other antibodies. Yet, the impact of the most recent bNAbs on the complement pathway has not been thoroughly analyzed.

Here, we investigated how bNAbs and polyclonal antibodies activate the complement at the surface of infected cells. Using Env-expressing cell lines and infected primary CD4 T cells, we measured C3 deposition and CDC induction. We report that only a subset of bNAbs, targeting the CD4 binding site and the V3 loop, triggers efficient C3 deposition. The Fc mutation E430G increases complement activity of HIV-1 bNAbs. Infected primary T cells treated with these bNAbs and decorated with C3 are not sensitive to CDC. The viral accessory proteins Vpu and Nef down-modulate Env levels and C3 deposition at the plasma membrane, whereas the cellular protein CD59 prevents rapid lysis of infected cells. However, complement-coated infected cells disappear within a few days of culture.

Results

Identification of complement-activating anti-Env antibodies

We selected a panel of 22 neutralizing (bNAb) and non-neutralizing (nnAb) antibodies targeting various epitopes of HIV-1 Env (Appendix Table S1) to study their ability to activate the complement pathway. We engineered a Raji B cell derivative stably expressing high levels of YU2-Env (Raji-Env cells). We chose Raji cells because (i) they lack CD4 and Env is not fusogenic in the absence of CD4, (ii) they do not express CD59, making them highly

sensitive to complement lysis, and (iii) treatment with the anti-CD20 antibody rituximab (RTX) triggers complement activation in Raji cells [45], providing a convenient positive control. Env present at the surface of Raji cells was correctly folded and functional, as demonstrated by the formation of syncytia after co-culture with CD4$^+$ T cells (not shown). As complement activation requires antibody binding, we first evaluated by flow cytometry the ability of our panel of antibodies to recognize Raji-Env cells (Fig 1A and B). We used RTX as a positive control. At 15 μg ml^{-1}, anti-Env antibodies bound to Raji-Env cells with different intensities, when compared to the isotype control (Fig 1A and B). The three anti-V3 and two of the CD4bs bNAbs (N6 and 3BNC117) displayed a high median fluorescence intensity (MFI) of binding (Fig 1B). The bNAbs targeting V1/V2, gp120/41 interface, and MPER, as well as the nnAbs were less efficient binders. The anti-HIV antibodies did not bind to parental Raji cells (not shown).

Complement activation was then evaluated by exposing the cells to antibodies and normal human serum (NHS) as a source of complement (Fig 1C–F). Cells cultivated without NHS or with heat-inactivated human serum (HIHS) were used as negative controls (Fig EV1A and B, and not shown). The activation of C3 was assessed by measuring C3b/iC3b deposition (later referred to as C3 deposition) by flow cytometry after 1 h (Fig 1C and D), and CDC was assessed by measuring the appearance of dead cells 24 h later (Fig 1E and F). The efficiency of C3 deposition varied with the antibodies, the more potent being those targeting the V3 loop, as well as some CD4bs and MPER bNAbs (Fig 1C and D). No detectable C3 deposition was observed in cells treated with antibodies and HIHS (Fig EV1A) or in the absence of anti-Env antibodies (Fig 1C and D). CDC induction was even more selective, as only bNAbs targeting the CD4bs and V3 loop triggered a large extent of cell death (Fig 1E and F). MPER bNAbs display no or limited CDC. Of note, m66.6, an anti-MPER antibody known to be polyreactive against self and non-self antigens [46], displayed C3 deposition in the absence of CDC. The two V1/V2 and the three gp120/41 interface bNAbs tested triggered neither C3 deposition nor CDC, despite a strong binding at the surface.

To further characterize these antibodies, we performed a dose–response analysis of their Env-binding and CDC activities (Fig EV1C). Both were dose-dependent, with EC50 varying with the antibodies. All antibodies plateaued at 15 μg ml^{-1}, with different levels of CDC (Appendix Table S2). We also visualized CDC in real time by time-lapse microscopy. The addition of propidium iodide (PI), which stains the nucleus of dying cells, allowed to follow the fate of Raji-Env cells. In the presence of 10-1074 and NHS, cell death occurred in most cells in about 3 h (Movie EV1).

We then sought to enhance the CDC activity of some of these antibodies by introducing a Fc point mutation (E430G) that favors hexamer organization and subsequent complement activation [47]. We chose two CDC-activating antibodies targeting distinct epitopes (3BNC117 and 10-1074) and one CDC-incompetent antibody (PGDM1400; Fig 1G). Introduction of the E430G mutation decreased the EC50 by threefold for both 3BN117 and 10-1074 (from 0.73 to 0.24 μg ml^{-1} and from 0.67 to 0.25 μg ml^{-1}, respectively), and increased the plateau of 3BNC117 (from 81 to 90% of cell death). The E430G mutation did not rescue CDC activation by PGDM1400, highlighting the inability of this antibody to trigger complement attack.
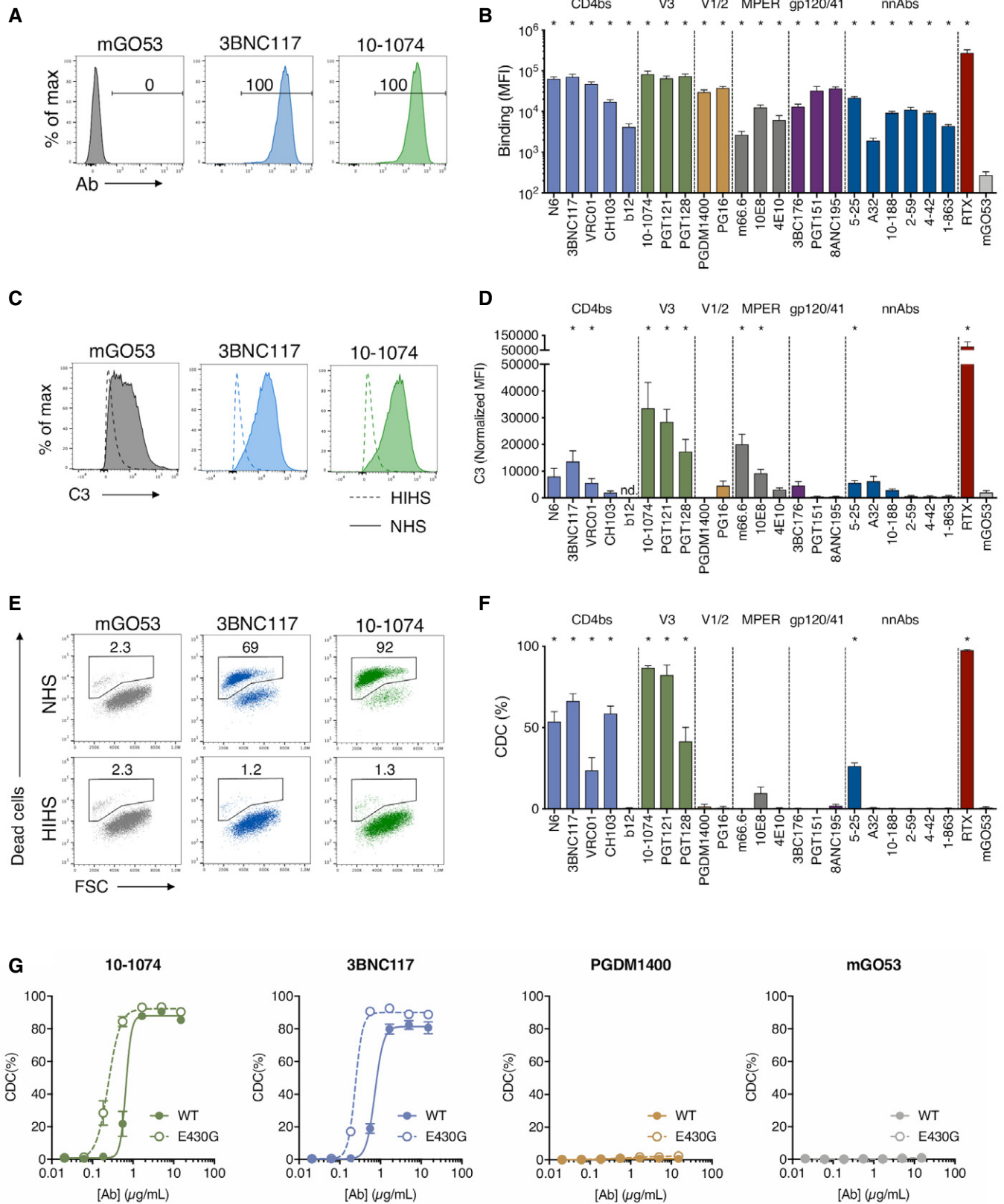
Figure 1.

Figure 1.   A subset of anti-HIV-1 antibodies triggers C3 deposition and CDC of Env-expressing cells.

A   Raji cells stably expressing HIV-1 YU-2 Env (Raji-Env) were stained with bNAbs (10-1074 or 3BNC117) or isotype control (mGO53) and analyzed by flow cytometry. The numbers indicate the % of positive cells. One representative experiment is shown.

B   Binding of the 24 indicated antibodies to the surface of Raji-Env cells. Results are expressed as the median fluorescence intensity (MFI) of staining. *n* = 6 independent experiments.

C   Raji-Env cells were incubated with indicated antibodies and either normal (NHS) or heat-inactivated (HIHS) human serum. After 1 h, surface levels of C3 were analyzed by flow cytometry. One representative experiment is shown.

D   C3 deposition by the 24 indicated antibodies. For each antibody, a normalized MFI is calculated by subtracting the MFI of the "no antibody" condition. (nd. not done). *n* = 3 donors of serum.

E   Raji-Env cells were incubated with indicated antibodies and either normal (NHS) or heat-inactivated (HIHS) human serum. After 24 h, cell death was determined by flow cytometry. The numbers indicate the percentage of dead cells. One representative experiment is shown.

F   CDC induction by the 24 indicated antibodies. The % of CDC was calculated as the relative percentage of dead cells compared to the "no antibody" condition. *n* = 3 donors of serum.

G   Raji-Env cells were incubated for 24 h with NHS and indicated concentrations of WT or E430G mutant bNAbs (10-1074, 3BNC117, or PGDM1400) or isotype control (mGO53). Cell death was measured by flow cytometry, and the % of CDC was calculated as the relative % of dead cells compared to the "no antibody" condition. *n* = 3 donors of serum.

Data information: Error bars indicate SEM. Significance was determined by comparing each antibody to mGO53. Only significant comparisons are depicted; **P* < 0.05, Mann–Whitney test.

Altogether, these results show that anti-Env antibodies differ in their capacity to activate the complement. Only eight out of 22 antibodies triggered efficient C3 deposition and CDC. The most potent bNAbs target the CD4bs and the V3 loop. Modifying the Fc region can increase the CDC activity.

C3 deposition at the surface of HIV-1-infected primary CD4 T cells

Raji-Env cells do not recapitulate the complexity of HIV-1-infected cells as they express high levels of Env and lack other viral proteins. Moreover, they do not express CD59, one key regulator of CDC. We thus measured complement activation at the surface of HIV-1-infected primary CD4 T cells. We first used the laboratory-adapted R5-tropic strain NLAD8. Based on the screen in Raji-Env cells, we selected antibodies that differ in their ability to activate the complement (3BNC117, 10-1074, and PGDM1400). We first measured by flow cytometry the capacity of these antibodies to bind the surface of HIV-1-infected (Gag$^+$) cells (Fig 2A). As previously reported [24,43], 3BNC117, 10-1074, and PGDM1400 efficiently recognized Gag$^+$ cells, and not bystander Gag$^-$ cells (Fig 2A). The antibodies stained more than 75% of infected cells (Fig 2A). The highest and lowest MFIs were obtained with 10-1074 and PGDM1400, respectively (Fig 2B). We next cultivated infected cells in the presence of antibodies and NHS to allow complement activation. We measured C3 deposition (Fig 2C and D) and MAC formation (C5b-9 deposition; Fig 2E and F) on Gag$^+$ cells by flow cytometry. We quantified CDC by assessing the disappearance of infected cells, as previously performed in ADCC assays (Fig 2G and H) [48]. 3BNC117 and 10-1074 triggered C3 and C5b-9 deposition at the surface of Gag$^+$ cells, when compared to controls (Fig 2D and F). Complement deposition was not detectable on bystander Gag$^-$ cells (Fig EV2A). PGDM1400, which did not trigger complement deposition in Raji-Env cells, was also inactive in T cells (Fig 2D and F). The frequency of infected cells similarly decreased after 24 h of culture with bNAbs and NHS or HIHS, likely reflecting the neutralizing activity of the antibodies (Fig 2G). We used the HIHS condition to calculate the relative disappearance of infected cells (see Methods). Using seven different donors of primary CD4 T cells, we did not detect CDC activity (Fig 2H). This absence of CDC was not due to an intrinsic resistance

of primary CD4 T cells, as anti-HLA-A/B/C (W6/32) and anti-CD52 (CAMPATH-1) antibodies triggered efficient CDC in non-infected cells (Fig EV2B and C). These two antibodies were also active in infected cells, indicating that HIV-1 infection does not render cells resistant to CDC (Fig EV2B and C).

Overall, these results show that anti-Env antibodies can trigger C3 deposition and MAC formation at the surface of primary HIV-1-infected CD4 T cells. Complement deposition by bNAbs does not lead to rapid cell lysis.

Env levels and Vpu regulate C3 deposition by bNAbs

HIV-1 regulates Env expression at the cell surface, limiting antibody binding and ADCC [48]. We thus hypothesized that Env levels may modulate C3 deposition and CDC. We generated Raji-Env clones stably expressing various levels of viral glycoproteins, as observed by the increase in the MFI of 10-1074 binding (Fig 3A). Of note, 10-1074 does not bind to parental Raji cells (Fig 3A). We measured the ability of 10-1074 (15 μg ml^{-1}) to trigger CDC in these cells (Fig 3B). The efficacy of C3 deposition and CDC increased with the surface levels of Env (Fig 3B and not shown). We selected three Raji-Env clones with low (D1), intermediate (D2), and high (D4) Env levels and compared these levels to those found at the surface of HIV-1-infected primary CD4 T cells (Fig 3C). Infected primary CD4 T cells exposed a lower amount of Env than the clone D1, which was poorly sensitive to CDC. This suggests that the quantity of Env exposed at the surface of infected T cells is too low to allow CDC induction by 10-1074.

The viral accessory protein Vpu eliminates the cellular protein tetherin/BST-2 from the membrane of infected cells. Tetherin retains budding viral particles at the cell surface, limiting viral spread and increasing recognition of infected cells by antibodies and ADCC [49–51]. Whether Vpu prevents C3 deposition or CDC is unknown. We thus compared antibody-mediated complement activation on CD4 T cells infected with WT or ΔVpu HIV-1 (NLAD8 strain). We tested the two most potent CDC antibodies, 10-1074 and 3BNC117, and their complement-enhanced E430G Fc variants. As expected, Vpu deletion increased binding of both antibodies at the cell surface (Figs 3D and EV3A). The E430G mutation did not influence antibody recognition of infected cells (Figs 3D and EV3A). However,
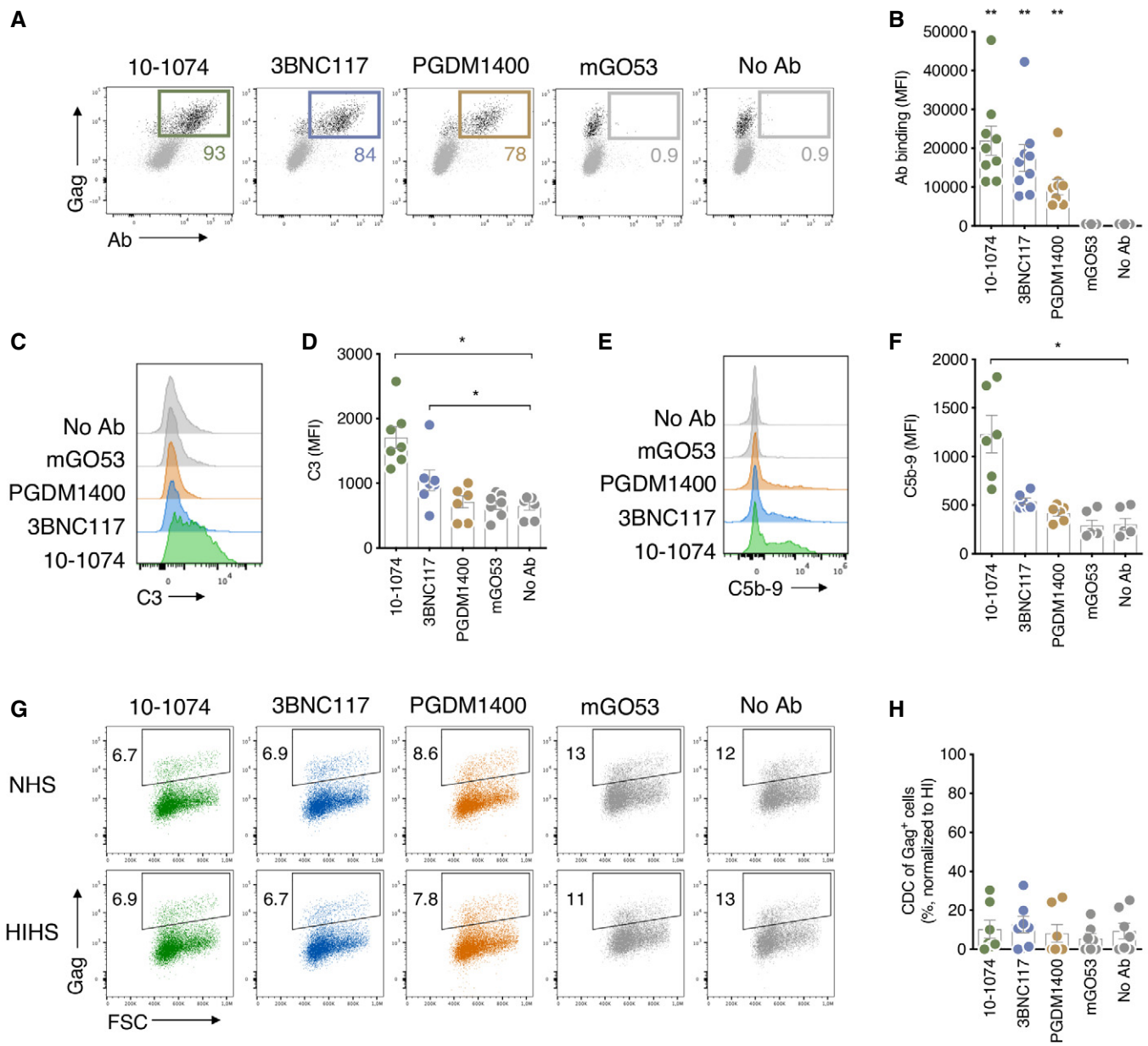
Figure 2. bNAbs allow complement deposition on HIV-1-infected CD4 T cells.

A Activated primary CD4 T cells were infected by HIV-1 (strain NLAD8) and subjected to antibody binding assay. The numbers indicate the % of Gag⁺ cells that are decorated by the indicated antibodies. One representative experiment is shown.

B Antibody binding at the surface of HIV-1-infected primary CD4 T cells. Results are expressed as median fluorescence intensity (MFI) of staining on infected (Gag⁺) cells. Each dot represents a single donor of CD4 T cells. n = 9 donors of CD4 T cells.

C HIV-1-infected (strain NLAD8) CD4 T cells were cultured with the indicated antibody and NHS. After 24 h, the surface levels of C3 on infected cells (Gag⁺) were determined by flow cytometry. One representative experiment is shown.

D C3 surface levels at the surface of HIV-1-infected primary CD4 T cells. Results are expressed as MFI of staining on infected (Gag⁺) cells. Each dot represents a single donor of CD4 T cells. n = 7 donors of CD4 T cells.

E HIV-1-infected CD4 T cells (strain NLAD8) were cultured with the indicated antibody and normal (NHS) or heat-inactivated (HIHS) human serum. After 24 h, the surface levels of C5b-9 (MAC) on infected (Gag⁺) cells were determined by flow cytometry. One representative experiment is shown.

F C5b-9 (MAC) surface levels on HIV-1-infected cells (strain NLAD8). Results are expressed as MFI of staining on infected (Gag⁺) cells. n = 6 donors of CD4 T cells.

G HIV-1-infected CD4 T cells (strain NLAD8) were cultured with the indicated antibody and NHS or HIHS to determine complement-mediated cytotoxicity. After 24 h, the percentage of infected (Gag⁺) cells in each condition was determined by flow cytometry. One representative experiment is shown.

H Complement-mediated cytotoxicity of HIV-1-infected CD4 T cells (strain NLAD8). For each antibody, the % of CDC was calculated at 24 h as the % of disappearance of Gag⁺ cells in NHS compared to HIHS. Each dot represents a single donor of CD4 T cells. n = 7 donors of CD4 T cells.

Data information: Error bars indicate SEM. *$P < 0.05$, **$P < 0.01$, Wilcoxon test. Only significant comparisons are depicted.
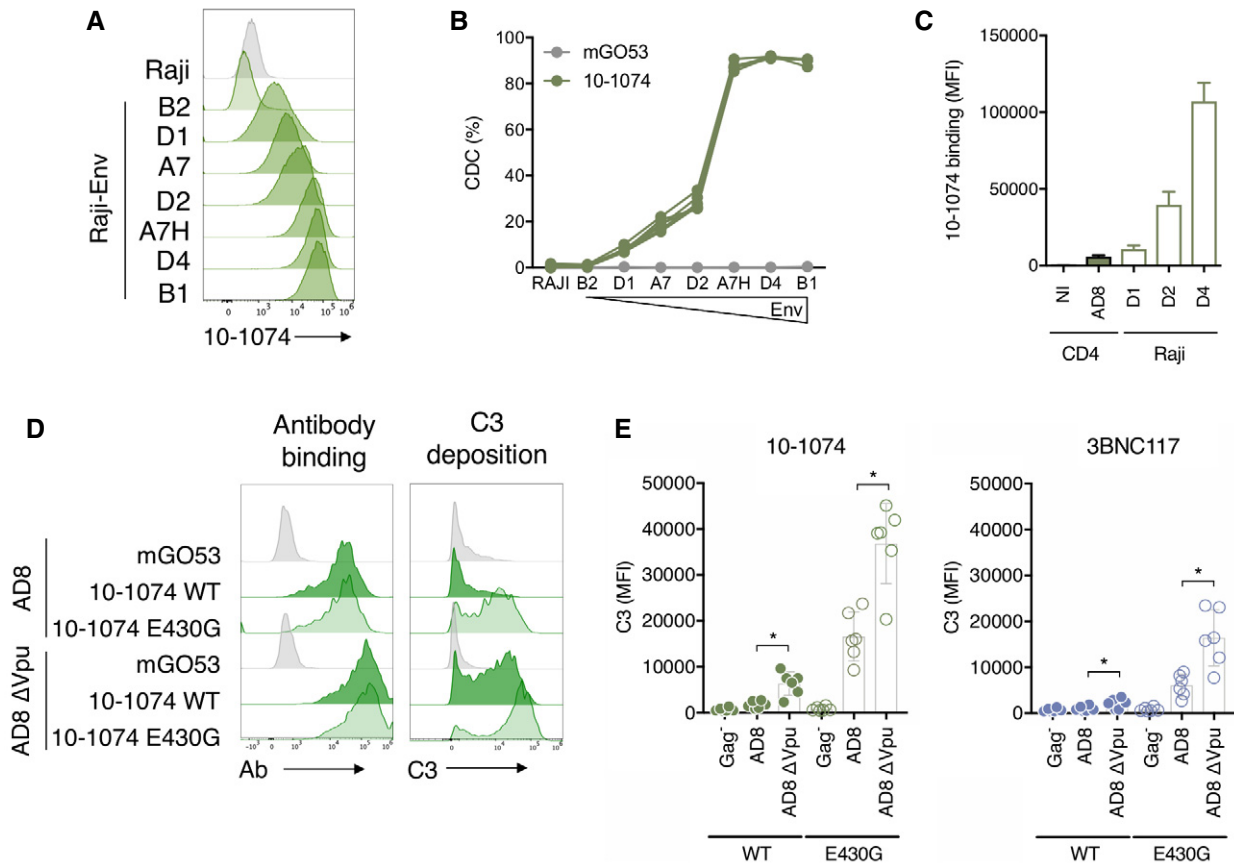
Figure 3. Low Env levels on HIV-1-infected CD4 T cells limit complement deposition.

A   Raji-Env cells were subcloned to obtain cell lines expressing increasing amounts of Env. Env surface levels were determined by the binding of 10-1074 and measured by flow cytometry. One representative experiment is shown.

B   Parental Raji cells and the different Raji-Env clones were cultured with 10-1074 or an isotype control (mGO53) and NHS for 24 h. The % of CDC was calculated as the relative percentage of dead cells compared to the "no antibody" condition. n = 3 donors of serum.

C   Non-infected (NI) and HIV-1-infected primary CD4 T cells (strain NLAD8) as well as three Raji-Env clones (D1, D2 and D4) were incubated with 10-1074, and antibody surface levels were analyzed by flow cytometry. Results are expressed as median fluorescence (MFI) of staining in total cells (Raji, n = 3 independent experiments) or in infected (Gag$^+$) cells (CD4 T cells, n = 5 donors).

D   HIV- or HIVΔVpu-infected CD4 T cells (strain NLAD8) were subjected to antibody binding with indicated antibodies, and surface levels were determined by flow cytometry (left panel). The staining obtained with mGO53 was similar to the background signal observed on unstained cells. Infected cells were cultured with the indicated antibodies and NHS for 24 h, and the C3 surface levels on infected (Gag$^+$) cells were determined by flow cytometry (right panel). One representative experiment is shown.

E   HIV- or HIVΔVpu-infected CD4 T cells (strain NLAD8) were incubated with NHS and either 10-1074 (left panel) or 3BNC117 (right panel). Both WT- or E430G-mutated antibodies were used. After 24 h, C3 surface levels on infected cells were analyzed by flow cytometry. Results are expressed as MFI of staining on infected (Gag$^+$) cells. The MFI of C3 staining on Gag$^-$ cells was included as a control. Each dot represents a single donor of CD4 T cells. n = 6 donors of CD4 T cells.

Data information: Error bars indicate SEM. *P < 0.05, Wilcoxon test. Only significant comparisons are depicted.

deletion of Vpu significantly increased C3 deposition, with either 3BNC117 or 10-1074 (2.1- and 3.5-fold, respectively; Fig 3D and E). The E430G mutation further increased C3 deposition, (six- and nine-fold, with 3BNC117 and 10-1074, respectively). The highest level of C3 deposition was triggered by 10-1074^{E430G} antibody against ΔVpu-infected cells (20-fold increase compared to the WT virus and a non-mutated antibody). Antibody binding correlates with C3 deposition (Fig EV3B). However, none of these conditions was associated with CDC (Fig EV3C).

Overall, these results show that the quantity of Env at the surface controls bNAb-mediated complement deposition. Vpu reduces Env levels and C3 deposition on infected cells.

CDC induction by anti-Env antibodies is counteracted by CD59

The complement regulatory protein CD59 inhibits the assembly of the MAC [52]. We observed CDC in Raji-Env cells that lack CD59, whereas CD4 T cells express high levels of CD59 (Fig 4A). Thus, we asked whether CD59 may prevent CDC of HIV-infected T cells. We knocked out CD59 in primary CD4 T cells using the CRISPR-Cas9 system. Seven days after CD59 CRISPR-Cas9 delivery, the knockout efficiency reached 67–97% when compared to control treatment (Fig 4A). The MFI of CD59 was identical to the isotype control, suggesting a complete elimination of the protein (Fig 4A). Control and CD59^{KO} cells were infected with WT or Vpu-deleted

NLAD8 and subjected to complement activation with 10-1074 or 10-1074^{E430G} (Fig 4B and C). No CDC was detectable in control HIV-infected cells (Fig 4C). In contrast, when CD59 was removed, CDC occurred with both WT and ΔVpu (Fig 4C). The highest level of CDC was achieved with ΔVpu-infected cells bound with 10-1074^{E430G} (up to 80% of elimination of infected cells). Of note, HIV-1 infection did not modulate CD59 surface levels (Fig 4D). Therefore, CD59 prevents CDC by bNAbs. The effects of CD59 and Vpu are additive.

Increased disappearance of infected T cells in the presence of complement

We next determined whether non-lytic complement deposition influences the fate of infected cells. To eliminate dying and bystander cells, we sorted infected cells. Primary CD4 T cells were infected with HIV-1 for 1 day, and the fraction of productively infected cells was enriched by magnetic depletion of non-infected (CD4^{high}) cells (Fig 5A). Enriched WT- or ΔVpu NLAD8-infected
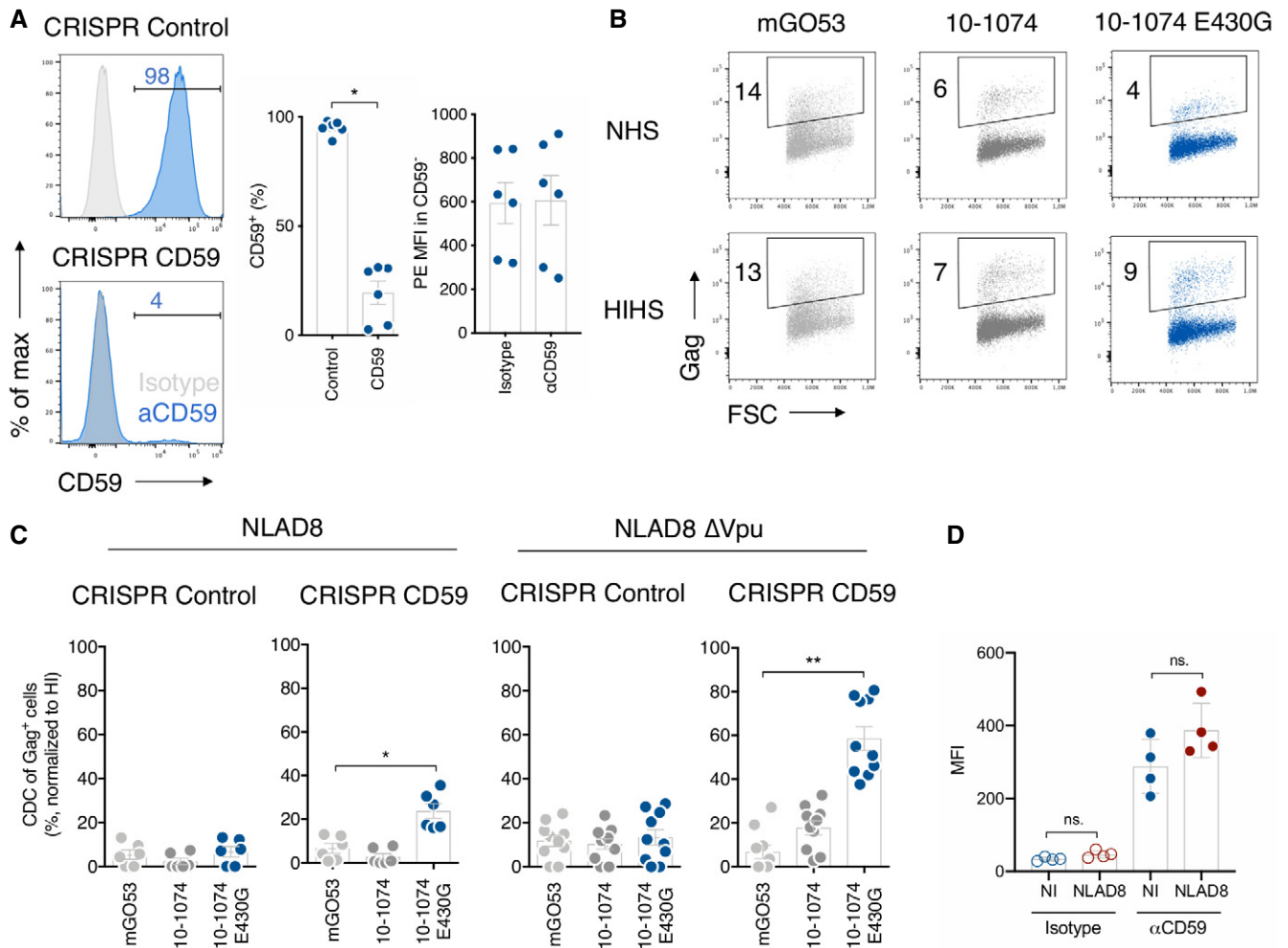

Figure 4. CD4 T-cell lysis by complement is restricted by CD59.

A Primary CD4 T cells were nucleofected with a control (CRISPR Control) or anti-CD59 (CRISPR CD59) guide RNA/Cas9 complex. CD59 surface levels were analyzed by flow cytometry 7 days after nucleofection. Isotype control (gray) and CD59 stainings (blue). One representative experiment is shown on the left. Numbers indicate the % of CD59$^+$ cells (middle) or the MFI of staining in CD59$^−$ cells (right). Each dot represents a single donor of CD4 T cells. n = 6 donors of CD4 T cells.

B NLAD8ΔVpu-infected CD59 KO primary CD4 T cells were cultured with indicated antibodies and NHS or HIHS for 24 h. Numbers indicate the percentage of infected (Gag$^+$) cells. One representative experiment is shown.

C CD59-KO (CRISPR CD59) or control (CRISPR Control) CD4 T cells infected with HIV WT (left) or HIVΔVpu (right; strain NLAD8) were cultured with the indicated antibody, and NHS or HIHS for 24 h. The % of CDC was calculated as the % of disappearance of Gag$^+$ cells after culture with NHS compared to HIHS. Each dot represents a single donor of CD4 T cells. n= at least 6 donors of CD4 T cells.

D CD59 surface levels on non-infected (NI) or HIV-1-infected (strain NLAD8) primary CD4 T cells were analyzed by flow cytometry. Results are expressed as the MFI of staining with an isotype control or the anti-CD59 antibody. n = 4 donors of CD4 T cells.

Data information: Error bars indicate SEM. *P < 0.05, **P < 0.01, Wilcoxon test. Only significant comparisons are depicted.

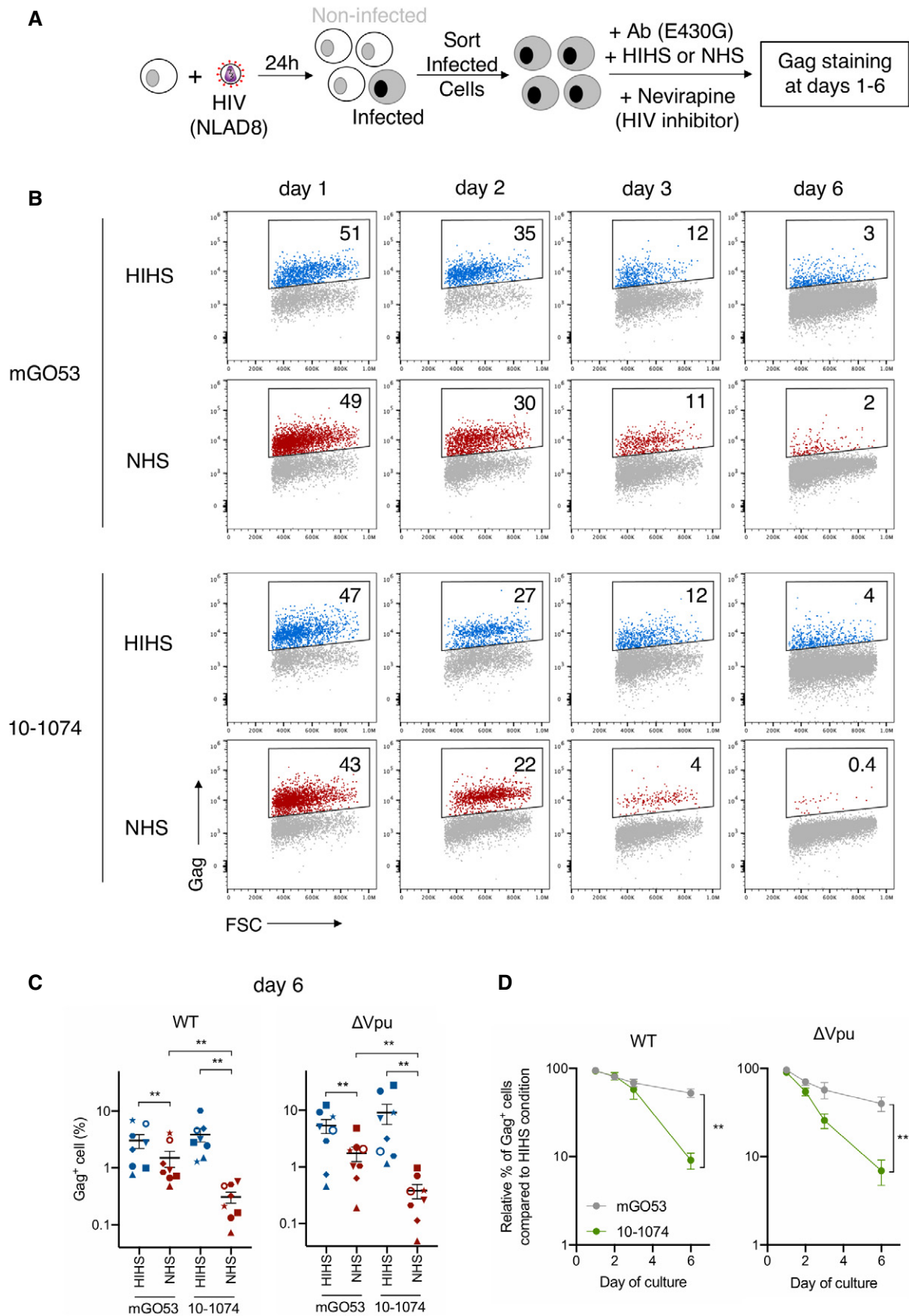Figure 5.

Figure 5. Increased disappearance of infected cells in the presence of complement.

A Primary CD4 T cells were infected with WT or ΔVpu HIV-1 (NLAD8 strain). After 24 h, productively infected cells were enriched by magnetic depletion of CD4^{high} cells, and cultured with the indicated E430G-mutated antibodies and with NHS or HIHS. The % of Gag^{+} was measured in live cells by flow cytometry at days 1, 2, 3, and 6.

B HIV-1-infected primary CD4 T cells were cultured with the indicated antibodies (E430G) and with NHS or HIHS. The frequency of infected cells was assessed by flow cytometry in live cells at days 1, 2, 3, and 6. Numbers indicate the percentage of infected (Gag^{+}) cells. One representative experiment is shown.

C Frequency of live HIV-1-infected cells (NLAD8 WT or ΔVpu strain) 6 days after incubation with the indicated antibodies, and with NHS or HIHS. Each donor of CD4 T cells is represented by a different symbol. n = 8 donors of CD4 T cells.

D Elimination of HIV-1-infected cells (strain NLAD8 WT or ΔVpu) over culture with the indicated antibody (E430G), and NHS or HIHS. The frequency of % infected (Gag^{+}) cells is determined by flow cytometry. The mean relative percentage of infected cells in NHS compared to the HIHS condition is depicted. n = 6–8 donors of CD4 T cells.

Data information: Error bars indicate SEM.; **P < 0.01; Wilcoxon test. Only significant comparisons are depicted.

cells (up to 90% of Gag^{+} cells) were then splitted into different cultures and incubated with either 10-1074^{E430G} or isotype control, in the presence of either NHS or HIHS. Cells were maintained in the presence of the reverse transcriptase inhibitor nevirapine to prevent viral propagation to remaining non-infected cells. The frequency of HIV-1-infected cells was then assessed in living cells (live/dead negative) at days 1, 2, 3, and 6 (Figs 5 and EV4A). NHS was not toxic and did not impact growth of uninfected CD4 T cells (Fig EV4B). As expected, a strong cytopathic effect was observed in all conditions and the frequency of Gag^{+} cells decreased overtime (Fig 5B). The frequency of HIV-1-infected cells was lower in cultures containing NHS, as compared to HIHS, suggesting that the complement by itself may impact the survival of infected cells. Moreover, in the presence of NHS, 10-1074^{E430G} strongly decreased the percentage of infected cells overtime, when compared to the isotype control (Fig 5B). At day 6 after treatment with 10-1074^{E430G} and NHS, the percentage of Gag^{+} cells was significantly lower than in control conditions (Fig 5C). At day 3, the relative percentage of Gag^{+} cells with 10-1074 in NHS compared to HIHS was lower with ΔVpu than with WT-infected cells (58 and 26% for WT and ΔVpu, respectively; P = 0.0312; Wilcoxon test; Fig 5D).

Altogether, these results suggest that the complement accelerates the disappearance of HIV-1-infected cells, through a mechanism that does not involve rapid CDC.

Primary HIV-1 strains are susceptible to complement deposition mediated by bNAbs

We next asked whether bNAbs may trigger complement deposition in cells infected with primary isolates of HIV-1. We infected CD4 T cells with two transmitted/founder HIV-1 strains (CH058 and

CH077) and a virus isolated from the reservoir of an HIV-1-infected individual (vKB18) [24] (Fig 6). We tested 10-1074, 3BNC117, and their E430G variants. The two bNAbs efficiently bound cells infected with CH058 and vKB18, while CH077-infected cells were preferentially recognized by 3BNC117 (Fig 6A). CH058-infected cells displayed complement deposition with 3BNC117^{E430G}, 10-1074, and to a higher extent with 10-1074^{E430G} (Fig 6B and C). The four bNAbs also triggered C3 deposition at the surface of vKB18-infected cells, with the highest values obtained with 10-1074^{E430G} (Fig 6C). With CH077-infected cells, C3 deposition was observed only with 10-1074^{E430G} and 3BNC117^{E430G} (Fig 6C). None of these conditions was associated with significant CDC (Fig 6D). Thus, lymphocytes infected by primary HIV-1 strains are targeted by bNAbs that instigate complement deposition.

Polyclonal anti-HIV antibodies activate the complement at the surface of infected cells and are counteracted by Vpu and Nef

We next determined whether antibodies present in patients' blood may activate the complement at the surface of infected cells. We tested sera from nine HIV-1-infected individuals under suppressive ART therapy (Appendix Table S3), and nine uninfected donors as controls. We first verified whether these sera harbor IgGs binding to HIV-1 infected cells. We selected the transmitted/founder strain CH058 because its Env is recognized by a wide array of antibodies [43]. The nine HIV^{+} sera recognized infected cells (Fig 7A). Moreover, complement deposition on CH058-infected cells was triggered by eight out of the nine sera (Fig 7B).

In most individuals, the anti-HIV-1 polyclonal antibody response consists of non- or poorly neutralizing antibodies that preferentially recognize non-functional or "open" conformation of Env (i.e.,

Figure 6. bNAbs trigger complement deposition on CD4 T cells infected with primary HIV-1 strains.

A Primary CD4 T cells infected with HIV-1 (transmitted/founder strains CH058 and CH077, and the primary strain vKB18) were incubated with the indicated bNAbs, and surface levels were analyzed by flow cytometry. The numbers indicate the % of bNAb^{+} cells among infected (Gag^{+}) cells. The percentages of infected cells were 27, 30, and 6% for CH058, CH077, and vKB18, respectively. One representative experiment is shown.

B CH058-infected CD4 T cells were cultured with the indicated WT or E430G mutant antibody and NHS. After 24 h, the C3 surface levels on infected (Gag^{+}) cells were determined by flow cytometry. One representative experiment is shown.

C CH058-, CH077-, and vKB18-infected primary CD4 T cells were incubated with the indicated antibodies and NHS. After 24 h, C3 surface levels on infected cells were analyzed by flow cytometry. Results are expressed as MFI of staining on infected (Gag^{+}) cells. Each dot represents a donor of CD4 T cells. n = 6 donors of CD4 T cells.

D Primary CD4 T cells infected with the indicated HIV-1 strains (CH058, CH077, or vKB18) were cultured with the indicated WT or E430G mutant antibodies and NHS or HIHS to determine complement-mediated cytotoxicity. After 24 h, the percentage of infected (Gag^{+}) cells in each condition was determined by flow cytometry. For each antibody, the % of CDC was calculated at 24 h as the % of disappearance of Gag^{+} cells in NHS compared to HIHS. Each dot represents a single donor of CD4 T cells. n = 6 donors of CD4 T cells.

Data information: Error bars indicate SEM. *P < 0.05, Wilcoxon test. Only significant comparisons are depicted.
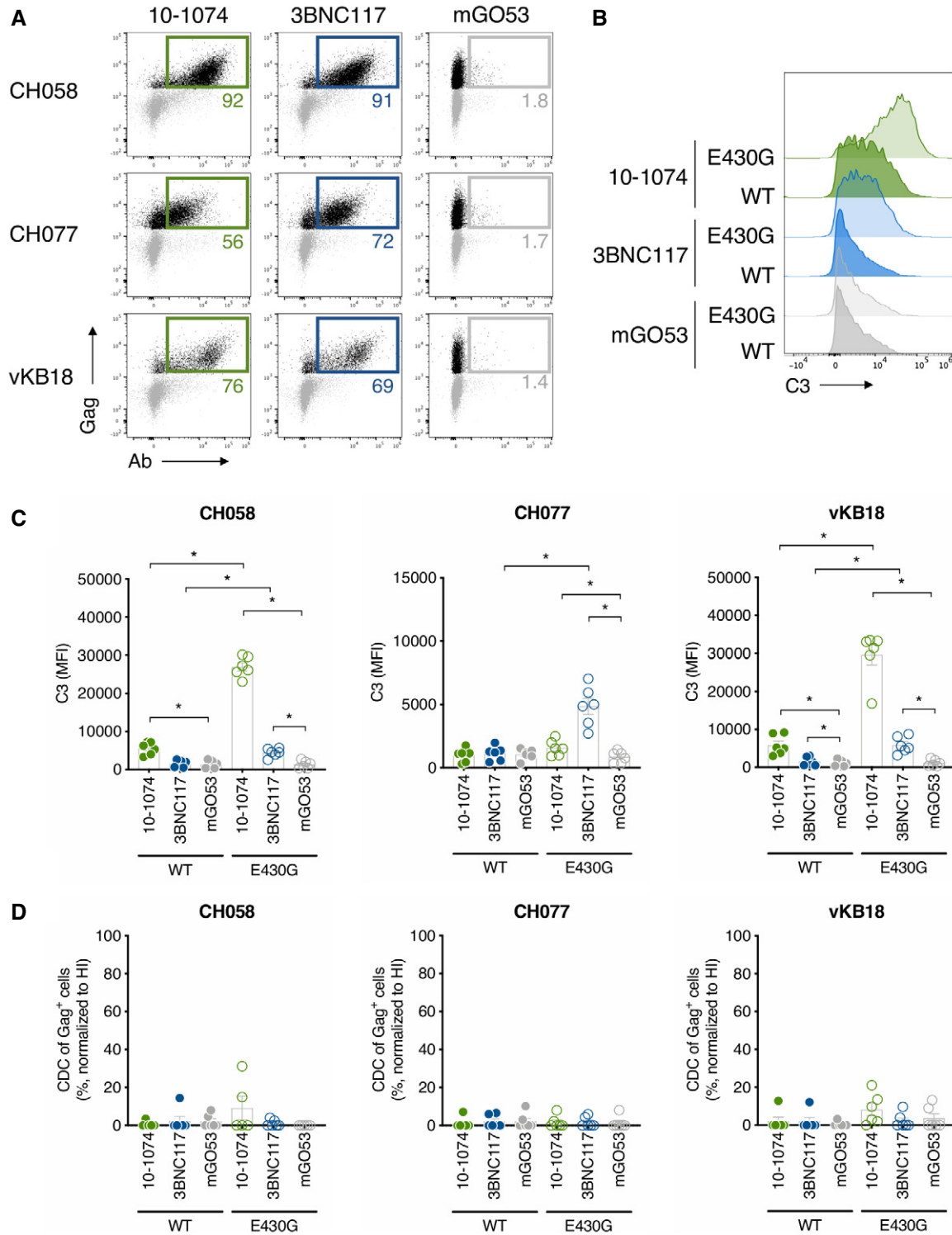
Figure 6.

bound to the receptor CD4) [53–55]. The viral protein Nef is an infectivity factor that down-regulates various cellular proteins from the surface of infected cells, including CD4, MHC-I, and SERINC3/5 [56]. Nef also modulates Env surface levels [57]. By promoting CD4 internalization, Nef limits Env-CD4 interactions at the membrane,

thus decreasing recognition by nnAbs and ADCC [48]. Nef synergizes with Vpu to counteract ADCC-mediated killing of infected cells [49,58]. Whether a similar mechanism exists for complement activation is unknown. To address this question, we infected CD4 T cells with CH058 WT, ΔNef, ΔVpu, and ΔNefΔVpu viruses. We then
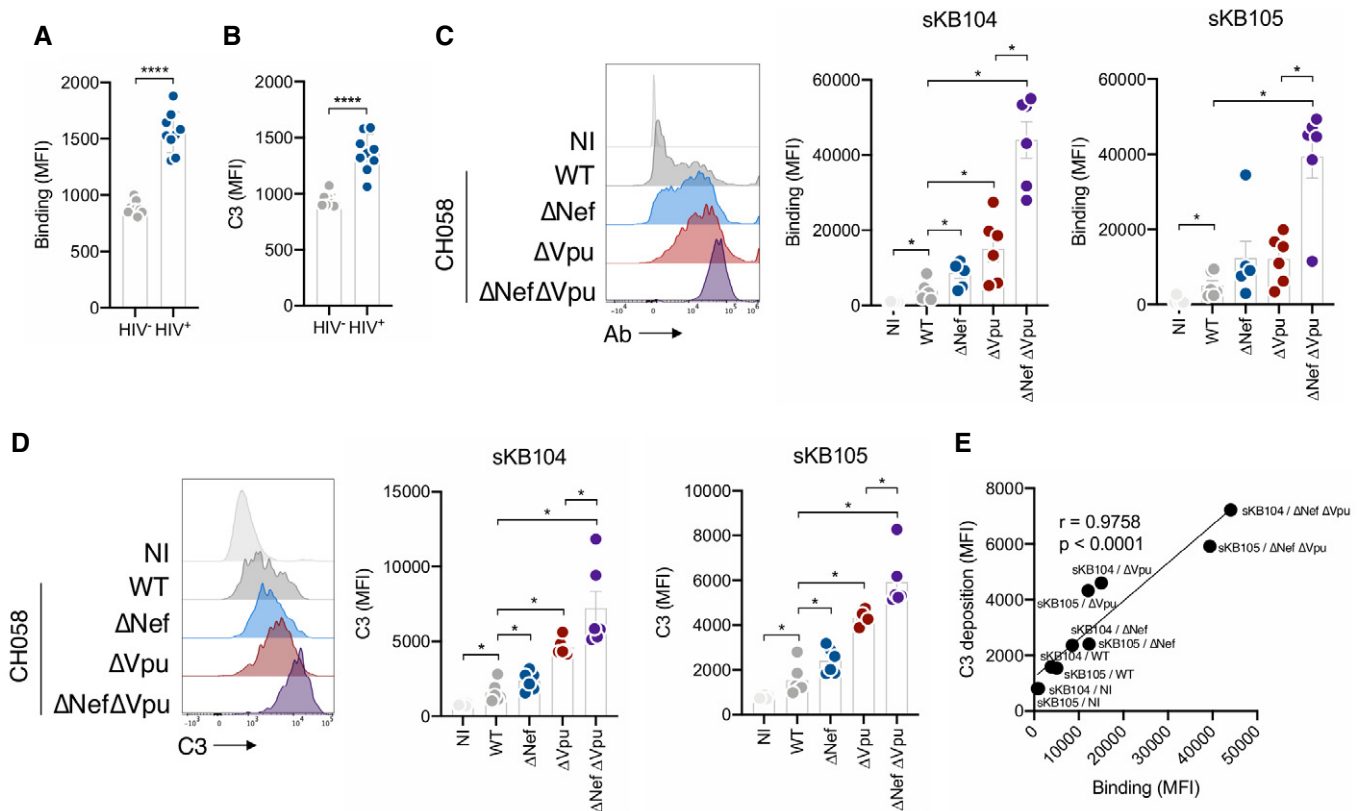
Figure 7. Vpu and Nef limit complement deposition by HIV-1-infected individuals' sera.

A HIV-1-infected primary CD4 T cells (strain CH058) were incubated with sera of HIV-negative (HIV⁻; n = 9) or ART-treated HIV-1-infected (HIV⁺; n = 9) individuals (serum dilution 1:1000). The levels of IgG binding on infected (Gag⁺) cells were analyzed by flow cytometry. Results are expressed as the MFI on infected (Gag⁺) cells. Error bars indicate SEM. ****P < 0.0001; Mann–Whitney test.

B CH058-infected primary CD4 T cells were cultured for 24 h with sera of healthy (HIV⁻; n = 9) or ART-treated HIV-1-infected (HIV⁺; n = 9) individuals (serum dilution 1:100) and 50% NHS from a healthy donor. C3 surface levels on infected (Gag⁺) cells were analyzed by flow cytometry. Results are expressed as the MFI on infected (Gag⁺) cells. Error bars indicate SEM. ****P < 0.0001; Mann–Whitney test.

C CD4 T cells not infected (NI) or infected with WT, ΔNef, ΔVpu, or ΔNefΔVpu HIV-1 (strain CH058) were incubated with the serum of HIV-1-infected ART-treated individuals (sKB104 or sKB105, dilution 1:1000). The levels of IgG binding on infected (Gag⁺) cells were analyzed by flow cytometry. One representative experiment is shown on the left. On the right, results are expressed as MFI of staining. Each dot represents a single donor of CD4 T cells. n = 6 donors of CD4 T cells. Error bars indicate SEM. *P < 0.05, Wilcoxon test. Only significant comparisons are depicted.

D Primary CD4 T cells either not infected (NI) or infected with WT, ΔNef, ΔVpu, or ΔNefΔVpu HIV-1 (strain CH058) were incubated with 50% NHS from a healthy donor and serum of HIV-1-infected ART-treated individuals (sKB104 or sKB105, dilution 1:100). The levels of C3 deposition on infected (Gag⁺) cells were analyzed by flow cytometry. One representative experiment is shown (left panel). Results are expressed as MFI of staining (right panel). Each dot represents a single donor of CD4 T cells. n = 6 donors of CD4 T cells.

E Correlation between patients' sera (sK104 and sKB105) antibody binding and C3 deposition on CD4 T cells either mock-infected or infected with WT, ΔNef, ΔVpu, or ΔNefΔVpu HIV-1 (strain CH058). Each dot is the mean of 6 donors of CD4 T cells. Correlation was analyzed by Spearman correlation. Correlation coefficient (r) and P-value are indicated.

Data information: Error bars indicate SEM. *P < 0.05, Wilcoxon test. Only significant comparisons are depicted.

measured polyclonal antibody binding and complement activation using the sera of two HIV-infected individual (Fig 7C and D). As previously observed, ΔNef- and ΔVpu-infected cells displayed higher antibody binding than WT-infected cells (Fig 7C). The double mutant ΔNefΔVpu displayed an even higher binding (Fig 7C). Complement deposition correlated with antibody binding, with higher C3 deposition on ΔNef- and ΔVpu-infected cells compared to the WT virus. This phenomenon was exacerbated on ΔNefΔVpu-infected cells (Fig 7D and E).

These data demonstrate that the polyclonal antibody response present in patients can trigger complement deposition. This process

is counteracted by Vpu and Nef, which both limit antibody recognition of HIV-1-infected cells.

Discussion

We report here that some bNAbs activate the complement at the surface of HIV-1-infected primary lymphocytes. In the presence of these bNAbs, infected cells display C3 deposition and MAC formation, but are not rapidly killed. CDC of infected lymphocytes is prevented by the low levels of Env at the surface, by the

constitutively expressed molecule CD59, and by the viral accessory proteins Vpu and Nef. Introduction of a point mutation in the Fc region (E430G) of bNAbs potentiates complement activation.

We show that the capacity to activate the complement is antibody-dependent and epitope-specific. Antibody binding to Env is necessary but not sufficient to activate the complement. Using 22 monoclonal antibodies, we show that bNAbs against the CD4bs and V3 loop glycans are more effective than those targeting the V1/V2 loop, the MPER, the gp41/gp120 interface, or non-neutralizing antibodies. Complement activation requires antibody hexamerization through Fc-Fc interactions [3]. It is thus likely that the antibody density and the accessibility or orientation of the Fc region dictate the potency of a given bNAb. V1/V2 glycan-targeting antibodies bind on the apex of the trimer, on a quaternary epitope that allows only one Fab to bind per trimer [59]. In contrast, the CD4bs and V3 loop authorize three Fab to bind each trimer [60,61], possibly facilitating Fc-Fc interactions. Further investigation will help uncovering the links that may exist between stoichiometry and orientation of antibodies binding to the viral envelope and their ability to trigger complement activation.

We also show that polyclonal antibodies present in the sera of infected individuals trigger C3 deposition, albeit less efficiently than bNAbs. Among the nnAbs tested in our study, only 5–25 (anti-gp41) triggers complement deposition and weak lysis in Raji-Env cells. Future work may help understanding whether the complement-inducing capacity of polyclonal antibodies is due to specific epitopes, or to cooperation between antibodies targeting various epitopes. Combining antibodies may increase complement activation as previously observed for anti-EGFR antibodies [62]. How bNAbs recognizing non-overlapping epitopes may synergize to promote CDC will deserve further investigation.

Envelope is present at the plasma membrane of infected cells to allow its incorporation into budding viral particles and to promote the formation of viral synapses with uninfected cells [63]. Recognition of Env by antibodies neutralizes viral cell-to-cell spread and render infected cells susceptible to ADCC [24,63]. Here, we further demonstrate that membrane-exposed Env may instigate complement deposition. Vpu limits Env surface levels, whereas Nef modulates Env conformation [48]. Nef, through its ability to down-regulate CD4 from the cell surface, counteracts ADCC mediated by non-neutralizing CD4-induced (CD4i) antibodies [49,58]. Our results uncover a novel function for Vpu and Nef, which limit Env exposure not only to escape ADCC, but also to avoid complement deposition.

We did not observe a rapid death of infected lymphocytes mediated by the complement pathway, even when the cells were opsonized by C3 and MAC. Knockout of CD59 by CRISPR-Cas9 restored sensitivity to killing, confirming previous results obtained with blocking anti-CD59 antibodies [40,41]. We further report that this non-lytic complement deposition leads to an accelerated disappearance of infected cells within a few days of culture. This may be due to a sensing of complement at the cell surface, leading to intrinsic signaling and modulation of cellular activation, metabolism, or growth kinetics [6–8]. Complement deposition may also trigger additional effects on infected cells, such as reducing infectivity of released virions [64] or increasing susceptibility to clearance by phagocytic cells [65]. Complement-dependent signaling through CD46 promotes a metabolic switch, with activation and contraction

of T-cell responses [66,67]. The mechanisms by which antibody-mediated complement deposition modifies the fate of HIV-1-infected lymphocytes will deserve further experimentation.

Hessell et al [23] reported that the Fc domain of the first-generation bNAb b12 is required for its prophylactic efficacy in macaques, independently of the complement pathway. We show here that b12 fails to activate the complement in Raji-Env cells, probably because its binding affinity to Env is not sufficient. This may explain why the complement does not contribute to b12 efficacy in vivo. Here, we identified a subset of second-generation bNAbs that trigger complement activation. Furthermore, the E430G mutation enhances the complement-activating capacity of 10-1074 and 3BNC117. Future experiments in animal models with such Fc-engineered antibodies may help understanding the role of complement in antibody efficacy in vivo.

The complement is a critical adjuvant of humoral immune responses [68,69]. Complement activity correlates with the appearance of broadly neutralizing antibodies in HIV-1-infected individuals and with the humoral response in vaccinees [70,71]. Complement-opsonized antigens are trapped by CR-expressing follicular DC within GC, allowing antigen presentation to B cells undergoing affinity maturation [70,72]. Recognition of iC3b and C3dg by CR2 on B cells decreases the threshold of BCR stimulation, favoring an optimal response [73]. Current clinical trials with bNAbs involve 3BNC117, VRC01, VRC07-523, N6, 10-1074, PGT121, PGDM1400, CAP256, 10E8, and derivatives [20]. Infusion of 3BNC117 improves immune responses and increases HIV-1 neutralization titers [17]. In our hands, 3BNC117, N6, and 10-1074 were more efficient at triggering complement activation than VRC01, PGDM1400, or 10E8. In humans, the antiviral effect of VRC01 is less marked than the two other antibodies. Future work will help understanding whether bNAbs that activate the complement display better therapeutic or prophylactic activities than other antibodies.

In conclusion, we demonstrate here that bNAbs and polyclonal anti-HIV-1 antibodies activate the complement at the surface of infected lymphocytes. Our study highlights how anti-HIV-1 antibodies use the complement and opens new avenues to exploit the complement system to fight HIV-1.

Materials and Methods

Cells

Raji and 293T cells were obtained from the ATCC (ATCC® CCL-86™ and ATCC® CRL-3216™) and tested negative for mycoplasma. Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood of healthy human donors from the Etablissement Français du Sang, in accordance with local ethical guidelines. CD4 T cells were obtained from PBMCs by positive immunomagnetic selection (Miltenyi) and activated for 3–5 days in the presence of IL-2 (50 IU ml^{-1}) and either T Cell TransAct™ (Miltenyi) or PHA (1 μg ml^{-1}; Oxoid). To generate Env-expressing cells, Raji cells were spinoculated (1,000 g for 1.5 h at 32°C) with a retroviral viral vector carrying Env (pMX-YU2 ENVΔCT-GFP-Puro^R) [74]. Transduced cells (GFP$^+$) were sorted and cultivated in the presence of puromycin (1 μg ml^{-1}; Sigma). Various levels of Env expression were then obtained by

subcloning. Briefly, limiting dilution in 96-well plates allowed for expansion of single cells. Clones were selected based on Env expression, as evaluated by measuring GFP and Env (using a 10-1074 staining; see below).

Viruses and infection

NLAD8 and transmitted-founder HIV-1 strains (CH058, CH077; obtained from the NIH AIDS Reagent Program) were prepared by the transfection of 293T cells along with vesicular stomatitis virus G to normalize infectivity. vKB18 was isolated from the reservoir of an ART-treated HIV-1-infected individual with undetectable viral load [24] and amplified on activated CD4 T cells from healthy individuals. No more than two passages were performed. Cells were infected for 4 h in the presence of HEPES and DEAE/Dextran, washed to remove unbound viral particles, and cultured in the presence of IL-2 (50 IU ml^{-1}) for up to 2–3 days before performing further experiments. Viral inocula were adjusted to achieve similar levels of Gag^+ cells at 48 h post-infection. When indicated, the fraction of live productively infected lymphocytes was enriched by magnetic depletion of non-infected, $CD4^{high}$ cells present in the population. Depletion was performed after 24 h of infection, using LD columns (Miltenyi) and CD4 microbeads (Miltenyi), according to the manufacturer's instructions. After sorting, up to 90% of the cells were CD4-negative, as controlled by flow cytometry.

Antibodies

Anti-Env monoclonal antibodies and the isotypic control mGO53 were produced as recombinant human IgG1 monoclonal antibodies by co-transfection of Freestyle 293-F suspension cells (Thermo Fisher Scientific) as previously described [61]. Antibodies were purified by batch/gravity-flow affinity chromatography using protein G sepharose 4 fast flow beads (GE Healthcare). The E430G point mutation was introduced in the IgG1 expression vector using the Quik-Change Site-Directed Mutagenesis Kit (Agilent Technologies). For biotinylation, antibodies were buffer-exchanged to Biotinylation Reaction Buffer (Quanterix) using an Amicon Ultra-0.5-ml filter (Merk). Biotin (EZ-Link NHS-PEG4-Biotin, Thermo Fisher) was added to the antibody solution at a molar ratio of 37.5 during 30 min at room temperature. Biotin in excess was removed with another Amicon dialysis step. Sera from ART-treated HIV-infected patients were also used at the indicated concentrations as a polyclonal source of anti-Env antibodies. Each participant provided written consent to participate in the study, which was approved by the regional investigational review board (IRB; Comité de Protection des Personnes Ile-de-France VII, Paris, France) and performed according to the European guidelines and the Declaration of Helsinki. Sera from healthy donors were used as controls. To measure complement deposition, cells were incubated with anti-C3/C3b/iC3b-APC (Clone 6C9; Cedarlane) or anti-C5b9 (clone aE11; Abcam) biotinylated in-house (see above) followed by streptavidin–Alexa Fluor 647 (Thermo Fisher).

Binding of antibodies at the cell surface

Cells (0.5–1 × 10^5) were incubated 30 min at 37°C with anti-Env antibodies or with an isotype human IgG1 control (mGO53) at 15 μg

ml^{-1} (unless otherwise stated), or with sera diluted 1:1,000. Cells were washed and incubated 30 min at 4°C with anti-human IgG1 (H + L) Alexa Fluor 647 (1:400 dilution; Life Technologies). Cells were then fixed with 4% PFA and stained for intracellular Gag with the anti-Gag KC57 murine monoclonal antibody as previously described [24]. Env-expressing Raji cells were stained using the same protocol, except that antibodies were biotinylated and revealed using a Alexa Fluor647-conjugated streptavidin (1:400 dilution; Life Technologies). Data were acquired on an Attune Nxt (Life Technologies) and analyzed using FlowJo software.

Complement activation assays

Cells (0.5–1 × 10^5) were cultivated in the presence of 50% normal (NHS) or heat-inactivated (HIHS) human serum and with or without antibodies (monoclonal antibodies at 15 μg ml^{-1}, unless otherwise stated, or patients' sera diluted 1:100). When indicated, nevirapine (NIH AIDS Reagent Program) was added to infected CD4 T-cell cultures at a final concentration of 12.5 μM. After 1 h (Raji) or 24 h (Raji and CD4 T cells), cells were incubated 30 min at 4°C with an APC-conjugated anti-C3 antibody to monitor complement activation at the cell surface (clone 6C9, Tebu-bio, final dilution 1:50). To measure CDC of Raji cells, the live/dead fixable aqua dead cell marker (1:1,000 in PBS; Life Technologies) was added for 30 min at 4°C before fixation. CDC was calculated using the following formula: 100 × (% of dead cells with antibody − % of dead cells without antibody)/(100 − % of dead cells without antibody). For CD4 T cells, cells were fixed with 4% PFA and stained for intracellular Gag. In some experiments the live/dead fixable aqua dead cell marker (1:1,000 in PBS; Life Technologies) was added for 30 min at 4°C before fixation. For each antibody condition, CDC was calculated as the complement-dependent disappearance of Gag^+ cells with the following formula: 100 × (% of Gag^+ cells in HIHS condition − % of Gag^+ cells in NHS condition)/(% of Gag^+ cells in HIHS condition). Negative values were set to zero.

CRISPR-Cas9 knockout in primary CD4 T cells

CRISPR-Cas9 knockout of primary CD4 T cells was adapted from [75,76]. To target CD59, three different crRNAs were used simultaneously (specific sequence: 5′-CAAGGAGGGTCTGTCCTGTT-3′, 5′-TAGGACAGTTGTAGCACTGC-3′, and 5′-GTTCTGTGGACAATCA CAAT-3′, all ordered from Integrated DNA Technologies, IDT). Pre-designed unspecific crRNA was used as control (IDT). crRNA and tracrRNA were resuspended in IDT Duplex Buffer according to the manufacturer's instructions. On the day of the nucleofection, duplexes were formed by mixing equimolar concentration of crRNA and tracrRNA, followed by 5-min annealing at 95°C. RNA duplexes were then mixed (1:2) with TrueCut™ Cas9 Protein v2 (Thermo Fisher) for 10 min at RT to generate ribonucleoprotein (RNP) complexes. Three days after activation with TransAct (see above), 2 × 10^6 CD4 T cells were resuspended in P3 Primary Cell Nucleofector™ Solution (Lonza), mixed with RNP and Alt-R® Cas9 Electroporation Enhancer (90 pmol, IDT), and nucleofected in a 4D-Nucleofector™ System (Lonza) using the P3 Primary Cell 4D-Nucleofector™ X Kit S (program FI-115). After nucleofection, cells were seeded in complete RPMI medium containing IL-2 and T Cell TransAct™. Seven days after nucleofection,

knockout efficacy was evaluated by flow cytometry using an anti-CD59 antibody (Miltenyi, final dilution 1:50).

Data processing and statistical analysis

Calculations were performed and figures were drawn using Excel 365 or GraphPad Prism 8.0. Statistical analysis was performed using GraphPad Prism, with Wilcoxon matched paired *t*-tests or Mann–Whitney unpaired *t*-tests. Spearman correlation coefficients (*r*) were calculated using GraphPad Prism. Scales were adjusted in each graph to highlight statistically significant differences.

Expanded View for this article is available online.

Author contributions

Conceptualization: JD, HM, OL, TB, OS; Methodology: JD, HM, TB, OS; Investigation: JD, FG-B, JB, VL, LG, ED, GM, HM, TB, OS; Resources: KB, OL; Writing—original Draft: JD, TB, OS; Writing—review & editing: JD, HM, OL, TB, OS; Funding acquisition: OL, HM, OS; Supervision: TB, OS.

Conflict of interest

The authors declare that they have no conflict of interest.

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