

The role of HIV-specific antibody-dependent cellular cytotoxicity in HIV prevention and the influence of the HIV-1 Vpu protein

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There is growing interest in the role of anti-HIV antibody-dependent cellular cytotoxicity (ADCC) antibodies in the prevention and control of HIV infection. Passive transfer studies in macaques support a role for the Fc region of antibodies in assisting in the prevention of simian-human immunodeficiency virus (SHIV) infection. The Thai RV144 HIV-1 vaccine trial induced anti-HIV ADCC antibodies that may have played a role in the partial protection observed. Several observational studies support a role for ADCC antibodies in slowing HIV disease progression. However, HIV evolves to escape ADCC antibodies and chronic HIV infections causes dysfunction of effector cells such as natural killer (NK) cells that mediate the ADCC functions. Further, four recent studies show that the HIV-1 Vpu protein, by promoting release of virions, reduces the capacity of ADCC antibodies to recognize HIV-infected cells. The review dissects some of the recent research on HIV-specific ADCC antibodies and discusses mechanisms to further harness ADCC antibodies in the prevention and control of HIV infection.

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Antibody-dependent cellular cytotoxicity and control of HIV infection

There has been a growing interest in HIV-specific antibody-dependent cellular cytotoxicity (ADCC) antibodies to prevent and modulate HIV. HIV-specific ADCC is mediated in large part by natural killer (NK) cells and requires aggregation of HIV antigen on the surface of infected cells. Other effector cells that are able to mediate killing of HIV-infected cells include monocytes and neutrophils [1–3]. HIV-specific antibodies that bind to both the HIV antigen (via their variable domains) and the FcγRIIIa (CD16) [4] (via their constant domain)

can induce specific activation of NK cells and other CD16⁺ cells [2]. This leads to NK cell degranulation and the release of the cytotoxic granules containing perforin and granzyme B and the liberation of cytokines/chemokines including interferon-γ (IFNγ) and tumour necrosis factor (TNF). The net effect is to kill antigen-expressing cells and create an antiviral state (Fig. 1).

Substantial evidence now supports the role for ADCC activity in the control of HIV infection, which subsequently results in a better disease prognosis in HIV-infected individuals [5–7]. ADCC antibody levels

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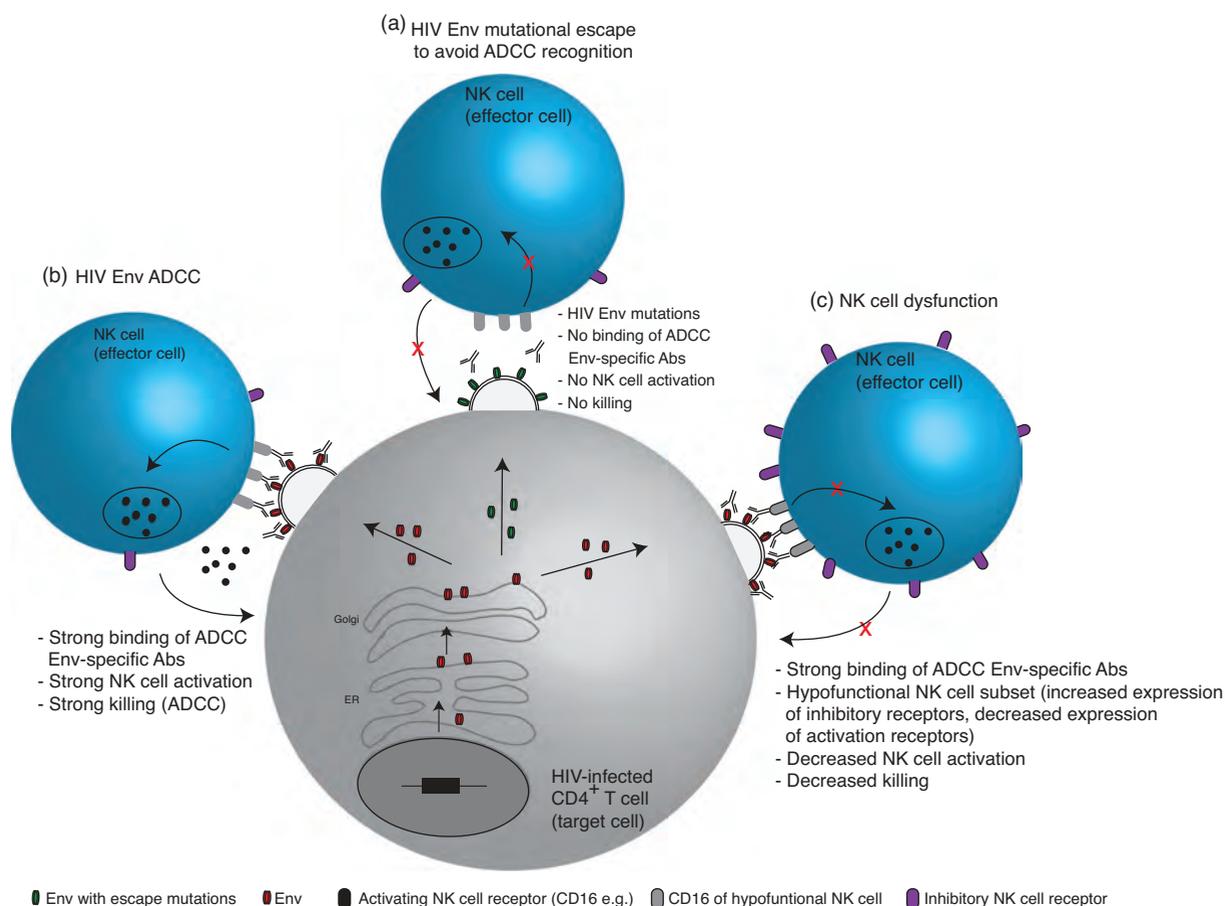


Fig. 1. HIV-specific antibody-dependent cellular cytotoxicity and avoidance of antibody-dependent cellular cytotoxicity recognition by natural killer cells. (a) Functional ADCC mediated by ADCC Abs binding to HIV Env that is present on budding virus. Decreased ADCC can be due to (b) HIV Env mutational escape to avoid recognition by ADCC antibodies. (c) NK cell dysfunction with increased expression of inhibitory receptors and decreased expression of activating receptors and an increase in a dysfunctional CD56-CD16⁺ subset. Blue cell: NK cell (effector cell), Grey cell: HIV-infected CD4⁺ T cell (target cells).

are important and correlate with slow progression of HIV infection [8–19] (Table 1).

Antibody-dependent cellular cytotoxicity as a correlate of protection in the RV144 trial

The Thai RV144 vaccine trial with its modest but significant protective immunity (31%, $P=0.04$) [20,21] has drawn further interest onto ADCC. The RV144 vaccine regimen did not induce broadly neutralizing antibodies (Nabs) or cytotoxic T-lymphocyte (CTL) responses but did induce robust HIV-specific ADCC responses [22].

Posthoc analyses showed that non-neutralizing antibodies to the C1 and V1/V2 regions of envelope (Env) correlated inversely with the risk of HIV infection [22]

and individuals who became infected also had escape mutations within V1-V2 Env sequences [23]. Further analyses showed that IgA antibodies elicited by the RV144 vaccine interfere with the binding of vaccine-induced IgG (mainly IgG1) and therefore diminish ADCC activity [24]. Indeed, low serum IgA but high levels of ADCC IgG were associated with a reduced risk of infection [20,22,25]. More recently, Yates *et al.* [26] demonstrated that the RV144 vaccine elicited V1-V2 specific IgG3, which also correlated with a decreased risk of HIV-1 infection, although this IgG3 response was not long-lived and could potentially explain the declining efficacy of the RV144 vaccine. Consistent with this finding, Chung *et al.* [27] showed that RV144 vaccination uniquely introduced a skewed and robust IgG3 antibody response. Although the vaccine used in the RV144 trial elicited lower antibody titres than the previous tested protein only vaccines, it introduced a robust polyfunctional antibody response, predominantly IgG1 and IgG3, which was able to simultaneously recruit multiple effector functions in a

coordinated manner [27]. These data suggest that as opposed to antibody titre and neutralizing activity alone, antigen-specific antibody function and subclass selection may provide a more informative measure of protective vaccine activity.

Passive transfer studies in animal models illustrate the potential importance of ADCC antibodies

Although it is difficult to definitively ascribe a protective role for vaccine-elicited ADCC antibodies from the RV144 trial, many studies suggest that ADCC antibodies play at least a partial role in protection of macaques against simian immunodeficiency virus (SIV) or simian-human immunodeficiency virus (SHIV) challenge and correlate with reduced peak viremia [17,28–32]. In macaques, ADCC-antibodies are mainly elicited against conformational epitopes [33], although some studies also identified ADCC antibodies to the V1 and V2 loops on Env after vaccination [34,35].

That IgG-mediated ADCC activity is important for protection has been previously demonstrated by Hessel *et al.* [36] who showed that ADCC activity is essential for the full protective efficacy of the b12 monoclonal neutralizing antibody. Using the LALA variant of b12 that contains a mutation in its constant region and abrogates Fc receptor signalling and ADCC while maintaining the ability to bind HIV-1 Env, a substantial reduction of the in-vivo protective capacity of b12 was detected [36]. The in-vivo protective effect of the 2G12 monoclonal neutralizing antibody was also partially mediated by non-neutralizing activities such as antibody-dependent cellular viral inhibition (ADCVI) [37]. The study of monoclonal neutralizing antibodies in a humanized mouse model also suggested that antibody Fc effector functions contribute to the inhibition of HIV entry [38].

Other in-vitro studies confirmed that the binding affinity of IgG to Fc receptors on different effector cells is essential and determines the magnitude of ADCC activity [39–41]. However, not all studies show this protective effect of ADCC activity. Two studies showed that passive transfer of non-neutralizing antibodies that had at least some ADCC activity *in vitro* was minimally effective in protecting macaques from SHIV infection [42,43]. Recent work in the influenza field has identified that NABs to conserved stem regions of the surface haemagglutinin protein that also mediate ADCC are potent inhibitors of infection [44,45]. There is likely to be an additive advantage of antibodies that are both neutralizing and mediate ADCC in protecting against viral infections compared with antibodies with either function alone.

Mutational escape to avoid HIV-specific antibody-dependent cellular cytotoxicity immunity

Both CTL and NAb against HIV lead to rapid selection for immune escape variants during HIV infection, which is one reason that HIV is difficult to control [46,47]. If ADCC antibodies exert pressure on HIV replication, we would expect to see mutational escape selected by ADCC antibodies also. Indeed, Chung *et al.* [48] mapped a series of linear ADCC epitopes, some of which were non-neutralizing epitopes, and showed evolution of sequences across several ADCC epitopes towards variants with reduced ADCC recognition. This study shows proof-of-principle of the in-vivo pressure applied by ADCC antibodies during HIV infection. It is also interesting to note that the HIV strains obtained from vaccinated individuals in the RV144 trial were enriched for variants of Env sequences [23] that may also reflect immune escape variants. Acharya *et al.* [49] recently performed a detailed structural analysis of the cluster of ADCC epitopes targeting the C1 region of Env implicated as a possible target of protective responses induced by the RV144 trial. Both antibody orientation and the ability to form multivalent antigen-antibody complexes were important determinants of ADCC potency [49].

Viral protein U inhibition of Tetherin reduces antibody-dependent cellular cytotoxicity recognition of infected cells

The cellular host restriction factor Tetherin/BST-2 is an IFN-inducible transmembrane protein that ‘tethers’ HIV particles on the surface of infected cells enabling the release of cell-free virus and therefore limiting viral spread [50,51]. HIV overcomes this restriction factor by expressing the accessory viral protein U (Vpu) that efficiently antagonizes Tetherin resulting in Tetherin downregulation and degradation [52–54] (Fig. 2a).

Four recent studies now have identified a major implication of the function for Vpu in limiting the presence of Env on the surface of infected cells. The ability of Vpu to diminish tetherin expression on the surface of infected cells reduces the binding and recognition of ADCC antibodies and thereby may protect infected cells from ADCC [55–58] (Fig. 2b).

Alvarez *et al.* [55] infected both a cell line, CD4⁺ Jurkat E6 T cells, and primary CD4⁺ T cells with either wild-type (wt) or with Vpu-deficient HIV (Δ Vpu) to investigate cell surface expression of Tetherin, the magnitude of ADCC antibody opsonization, the level of Fc γ RIIIa signalling and CD107a degranulation and killing by NK cells. Surface expression of Tetherin was

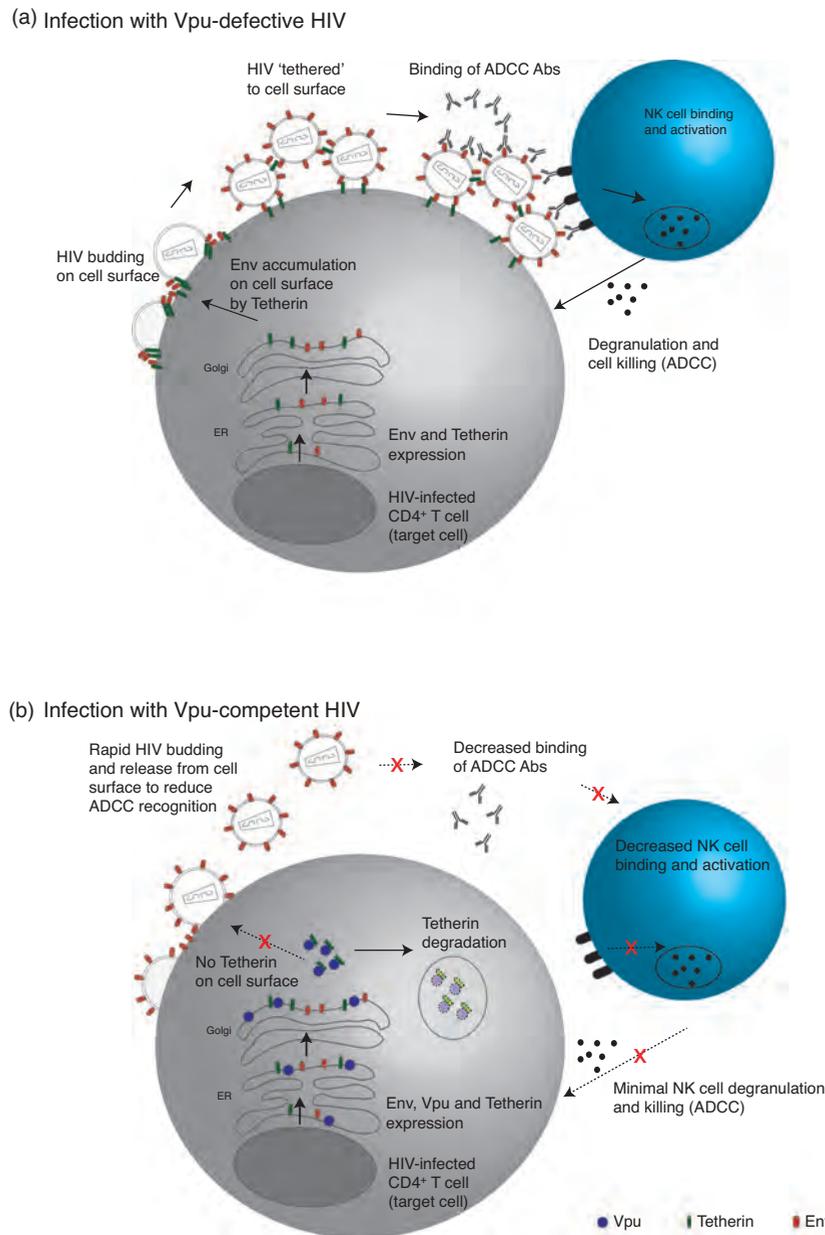


Fig. 2. The role of viral protein U in preventing HIV-specific antibody-dependent cellular cytotoxicity. (a) The release of HIV is prevented by Tetherin in the absence of Vpu. ADCC-mediated antibodies that bind to HIV tethered to the surface of HIV and are targets for strong ADCC mediated through effector cells such as NK cells. (b) In the presence of Vpu, Tetherin is downregulated and degraded leading to rapid release of HIV virions, decreased expression of Env on the cell surface and therefore decreased binding of antibodies that mediate ADCC. Blue cell: NK cell (effector cell), Grey cell: CD4⁺ T cell (target cells).

significantly increased in cells infected with Δ Vpu, but not wt HIV. Increased levels of Env were detected on the surface of Δ Vpu HIV-infected cells, which resulted in increased antibody opsonization by the monoclonal NAbs b12, 2G12 and 4E10 as well as serum antibodies from HIV-infected individuals. The binding of anti-HIV antibodies correlated nicely with the Tetherin surface expression. Further, surface Tetherin expression and antibody binding correlated with higher levels of Fc γ RIIIa signalling suggesting that Tetherin-mediated

Fc γ RIIIa stimulation is dependent on the engagement of the antibody Fc region. An increase in both the level of NK cell degranulation and killing of infected cells was detected in the absence of Vpu compared with wt HIV-1 infected cells. The authors further confirmed that the lack of tetherin was the primary reason that cells failed to activate Fc γ RIIIa signalling by both complexing surface Tetherin and using Vpu mutants. Interestingly, Tetherin-mediated activation of Fc γ RIIIa signalling was also increased by b12 antibody variants that are known to

enhance the binding to Fc γ RIIIa as previously described [39].

Overall, these data imply that Tetherin could play a major role in enhancing the otherwise limited expression of HIV Env on the surfaces of infected cells. Further, the absence of viral aggregates in the presence of Vpu or when surface Tetherin is not expressed results in the enhanced binding of antibodies, which may further enhance ADCC by acting as a directed mechanism for Fc γ RIIIa signalling.

A study by Arias *et al.* [56] published at around the same time independently showed similar results. The deletion of Vpu resulted in an increased susceptibility of infected cells to ADCC. Using an in-vitro infection and ADCC model, the authors showed that cells infected with different strains of Δ Vpu HIV are 60-fold more susceptible to antibody-mediated killing in the presence of pooled HIV immunoglobulin (HIVIG) than cells infected with the wt HIV equivalent. Similar to the study by Alvarez *et al.* [55], increased susceptibility to ADCC was correlated with increased Env expression and increased Tetherin expression on the cell surface. In the context of ADCC mediated by pooled HIVIG, Arias *et al.* [56] suggest that Vpu-mediated downregulation of CD4 [59] or NK, T and B-cell antigen (NTB-A, a costimulatory molecule required for NK cell activation [60]) did not reduce susceptibility to ADCC. However, there are many CD4-induced epitopes that are ADCC target and Pham *et al.* (see below [57]) and recently Veillette *et al.* [61] show the importance of CD4⁺-induced ADCC epitopes that are disrupted when CD4⁺ is downregulated by Nef and/or Vpu. Mutations within Vpu that completely impair Tetherin antagonism dramatically increased susceptibility to ADCC to a similar extent as the complete deletion of Vpu. In contrast, HIV with a Vpu mutation that interferes with CD4⁺ binding only had a small affect on the susceptibility of virus-infected cells to ADCC. Again, the effects of these mutations on ADCC also correlated with the surface expression of Env and Tetherin. The authors also demonstrated that RNAi-knockdown of Tetherin, but not CD4⁺ or NTB-A, significantly increased the resistance of HIV-infected cells to ADCC.

The third article by Pham *et al.* [57] also investigated whether the depletion of CD4⁺ and Tetherin negatively affected ADCC function. This study was done using the ADCC-competent monoclonal NAb 2G12 and A32. A32 recognizes an epitope highly dependent on the cell surface expression and interaction of CD4⁺ and Env. Enhanced binding of both 2G12 and A32 to Δ Nef or Δ Vpu and even more to Δ Nef Δ Vpu HIV-infected cells was observed. Interactions between CD4⁺ and Env in infected cells exposed the desired A32 (ADCC) epitope on cell-surface Env molecules, marking infected T cells for lysis by immune cells. Indeed, enhanced binding of

A32 resulted in increased susceptibility to ADCC. This was most evident in T cells infected with the Δ Nef Δ Vpu virus. Similar to the other two studies, Pham *et al.* [57] showed that increased cell-surface Env density due to Tetherin further enhanced the efficiency of ADCC. When Tetherin was partially depleted from CEM.Nkr T cells using a Tetherin-targeting shRNA, the level of released Δ Vpu-HIV particles was restored to wt HIV levels. This highlights the independent contributions of CD4⁺ and Tetherin on ADCC epitope exposure. The intensified susceptibility of T cells infected with HIV lacking Nef and/or Vpu to ADCC was recapitulated when plasma samples from HIV-infected patients were used instead of mAbs. The Env recognition patterns by patient plasma samples nearly mirrored those by A32 and ADCC activity was most robust in Δ Nef Δ Vpu-HIV-infected targets.

Lastly, a study by Li *et al.* [58] also showed that in contrast to the direct antiviral effects of Tetherin by modulating cell surface expression of virus protein, the immunomodulatory effects are linked to the endocytosis of the molecule. Using the model lentivirus Friend Retrovirus, they demonstrated that C57BL/6 mice encoding endocytosis-competent Tetherin exhibited lower viremia and Friend Retrovirus induced disease postinfection than mice encoding endocytosis-defective Tetherin. Protection also correlated with stronger NK cell responses and virus-specific CD8⁺ T-cell responses. The results demonstrate that Tetherin acts as a modulator of the cell-mediated immune response against retrovirus infection in an in-vivo mouse model.

To conclude, all four of these recently published studies implicate Vpu antagonism of Tetherin as an ADCC evasion mechanism that prevents antibody-mediated clearance of virally infected cells [55]. By serving as a link between innate and adaptive immunity, the antiviral activity of Tetherin may be augmented by virus-specific antibodies, much greater than previously appreciated [56]. This suggests a new mechanism by which HIV Vpu function to protect infected cells from ADCC and promote viral persistence [57]. The findings suggest that ADCC recognition of infected cells, and the evasion of this process via the Vpu-Tetherin interaction, could be a relatively potent immune response if it can be effectively harnessed [62].

Convergent interest in viral protein U, antibody-dependent cellular cytotoxicity and natural killer cells

An involvement of Vpu in antiviral immunity mediated by NK cells has also been reported from different directions. Wren *et al.* [13] showed that Vpu can actually be a target of ADCC immunity, with a significant

minority of HIV-infected individuals with slow HIV progression targeting linear epitopes in Vpu with ADCC antibodies (six of 65 individuals, 11%) compared with individuals with progressive HIV infection (0 of 74 individuals). The Vpu protein spans the cell membrane of HIV-infected cells so it is conceivable that Vpu-specific ADCC immune responses may disable Vpu-mediated Tetherin inhibition thus enhancing Env-specific immune responses and contributing to slower progression of HIV infection. We have previously demonstrated (data unpublished) that a small aliquot of ADCC-mediating Vpu antibody extracted from plasma of a long-term slow progressor can inhibit HIV replication *in vitro*, although we have not established the mechanism by which this occurs (i.e. Tetherin inhibition or a direct anti-Vpu effect). In addition, Alter *et al.* [63] have described mutations within Vpu that are linked to specific recognition of Vpu-expressing cells by subsets of NK cells. This 'direct' killing of Vpu-expressing cells by NK cells (in the absence of ADCC Abs) appears to be another mechanism (in addition to CTL immunity; [64–66]) whereby Vpu modulates antiviral immunity. Taken together, Vpu targeting by anti-HIV immune responses appears common and may reflect a site of vulnerability within the virus. Although a somewhat variable protein, it will be interesting to assess whether targeting Vpu by vaccination is an effective strategy. Targeting Vpu by vaccination could conceivably force mutations in Vpu that partially abrogate the ability of Vpu to inhibit ADCC responses, as shown in the mutational analyses described above [55–57].

Summary and future directions on HIV-specific antibody-dependent cellular cytotoxicity research

ADCC is an important immune response against HIV. These new data indicate that Vpu and its ability to counteract tetherin is important in the protection of HIV-infected cells from ADCC by reducing the expression of HIV Env on the surfaces of infected cells. In the presence

of Vpu–Tetherin interaction, antibody binding is decreased that results in reduced ADCC. Thus, the antiviral activity of Tetherin may be much greater than previously appreciated based on its ability to inhibit virus release *in vitro*. Anti-Vpu drugs or RNAi that target the anti-Tetherin activity of Vpu may result in inhibitory effects on virion detachment and increased potency of ADCC directed against virus-infected cells and therefore achieving a substantial reduction in HIV-1 replication. Thus, future studies should focus on the development of anti-Vpu therapeutics to release Tetherin's full potential to enhance ADCC *in vivo*. Although these recent studies only looked at Vpu's effect on other ADCC, Env-specific antibody-effector functions such as antibody-dependent phagocytosis or complement fixation might also be expected to be diminished by the Vpu–Tetherin interaction.

One open question is why ADCC inversely correlates with HIV disease progression if Vpu is so effective at blocking ADCC recognition of infected cells. A possible reason is that Env sheds from infected cells that may bind to neighbouring uninfected T cells via CD4⁺ interaction. If these 'Env-coated' cells are then opsonized by antibodies, they are most likely activating NK cells. This in turn would lead to cytokine secretion that may recruit other immune effector cells into that area of viral replication and creates an antiviral state.

The importance of the Vpu–Tetherin interaction and its influence on ADCC recognition reveals a very limited knowledge on the kinetics of virion release *in vivo*. Without knowing the fine kinetics of how quickly virions are released, it is hard to say whether ADCC antibodies could bind to Env on the budding virus. In addition, the amount of Env presented on infected cells during the budding process before virions are released is unknown but likely to be small. There could be a short window of opportunity wherein ADCC antibodies bind to the Env on the budding virus or simply to membrane-associated Env protein that is not incorporated into budding virus at that time. This antibody binding could then mediate ADCC. It may require relatively high levels of ADCC antibodies to be present to facilitate rapid recognition of

Table 1. Evidence of antibody-dependent cellular cytotoxicity in protection or control of HIV.

Evidence of ADCC in protection or control of HIV	Comment	Refs
RV144 correlates analyses	Primary correlates analysis showed positive correlation with V1-V2 Env antibodies and inverse correlation with protection with IgA Env antibodies with a risk of infection. Secondary analysis has implicated ADCC.	[22,24,27]
Passive transfer studies	Macaque passive transfer studies with Fc-mutated Nabs implicate role for Fc functions in protection from SHIV. Mouse HIV entry studies also implicate Fc functions as being important. Macaque passive transfer with non-Nabs have not shown protection from SHIV, although ADCC potency of transferred antibodies is unclear.	[36–38,42,43]
Mutational escape from ADCC during HIV infection	Mapped linear ADCC epitopes in Env undergo mutations during HIV infection that reduce ADCC recognition, implying immune pressure being applied by ADCC antibodies.	[48]
Association of ADCC with slowed HIV progression	Several groups show HIV-specific ADCC associated with slowed progression.	[5–19]

infected cells prior to virion release. Current studies in our laboratory are underway to identify B-cells secreting Vpu-specific ADCC antibodies in an effort to clone and produce these antibodies to characterize their ability to inhibit HIV replication *in vitro* and *in vivo*.

Future studies on anti-Vpu drugs and the *in-vivo* investigation of Tetherin's ADCC enhancing activity in different patient cohorts will shed more light onto this matter. Nonetheless, future studies should focus on how to induce potent and specific ADCC-mediating antibodies, as they are the essential key to any antibody-effector function.

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Conflicts of interest

The authors have no conflicts of interest.

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