Beyond Viral Neutralization

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Abstract

It has been known for more than 30 years that HIV-1 infection drives a very potent B cell response resulting in the production of anti-HIV-1 antibodies targeting several viral proteins, particularly its envelope glycoproteins (Env). Env epitopes are exposed on the surfaces of viral particles and infected cells where they are targets of potentially protective antibodies. These antibodies can interdict infection by neutralization and there is strong evidence suggesting that Fc-mediated effector function can also contribute to protection. Current evidence suggests that Fc-mediated effector function plays a role in protection against infection by broadly neutralizing antibodies and it might be important for protection by non-neutralizing antibodies. Fc-mediated effector function includes diverse mechanisms such as antibody-dependent cellular cytotoxicity (ADCC), antibody-mediated complement activation, antibody-dependent cellular phagocytosis, antibody-dependent cell-mediated virus inhibition, antibody-mediated transcytosis inhibition, and antibody-mediated virus opsonization. All these functions could be beneficial in fighting viral infections, including HIV-1. In this perspective, we discuss the latest developments in ADCC research discussed at the HIVR4P satellite session on non-neutralizing antibodies, with emphasis on the mechanisms of ADCC resistance used by HIV-1, the structural basis of epitopes recognized by antibodies that mediate ADCC, natural killer-cell education and ADCC, and murine models to study ADCC against HIV-1.

Keywords: HIV-1, Env, ADCC, non-neutralizing antibodies, neutralization, humanized mouse models, Fc, KIR, NK

Structural Characterization of Envelope Glycoprotein Antibody-Dependent Cellular Cytotoxicity Epitopes

TRANSITIONAL, DISCONTINUOUS EPITOPES in the A32-subregion of HIV-1 gp120 (cluster A epitopes) are targets for humoral responses that involve Fc receptor (FcR)–dependent immune functions without conventional neutralization activities.^{1–3} Our recent studies described the A32-subregion at the atomic level by providing the A32 epitope footprint and model of how these envelope glycoprotein (Env) epitopes are en-

gaged into the effective antigen–antibody–Fc-receptor immunocomplexes that lead to potent antibody-dependent cellular cytotoxicity (ADCC).^{4,5} The A32-subregion maps to the discontinuous site involving residues of mobile layers 1 and 2 of the inner domain within the constants 1 and 2 (C1–C2) region of gp120 in its CD4 receptor-bound state. This region is buried at the gp120–gp41 interface, comprising part of the gp41 docking site in the Env trimer present on the surface of free viral particles or productively infected cells. It requires a series of structural rearrangements of the Env spike for

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effective exposure, which *in vivo* is induced by the triggering of the Env trimer with cell surface CD4. Recent work indicates that in addition to cell surface CD4,³ forcing Env to sample the CD4-bound conformation using small CD4 mimetic compounds (CD4mc)⁶ in conjunction with coreceptor binding site (CoRBS) antibodies is sufficient to expose these epitopes and sensitize HIV-1-infected cells to ADCC mediated by antibodies recognizing this region.⁷ Additional work showed that CD4mc enhances viral neutralization and ADCC activities by antibodies elicited in nonhuman primates by several different Env immunogens⁸ suggesting that combining a vaccine with CD4mc, administered orally or in a microbicide formulation, might be useful as a prophylactic strategy against HIV-1 transmission.

Since the A32-subregion is highly conserved among HIV-1 isolates^{5,9,10} and is targeted by antibodies that do not require high levels of somatic mutation for potency, it might represent a promising target for C1/C2 monoclonal antibody (mAb)-based immune therapy either alone^{11,12} or in combination with CD4mc. Accordingly, there is a strong prediction that ADCC responses specific for A32 epitopes may be cross-reactive since critical contact residues forming these epitopes, such as W69, are extremely well conserved^{5,10} due to their role in maintaining Env stability,^{5,9,10} thus suggesting that these epitopes will undergo limited immune escape.

A recent comparison of HIV-1 Env-specific antibodies of diverse specificities revealed that ADCC generally correlates with neutralization.¹³ While non-neutralizing antibodies to CD4-induced (CD4i) epitopes of gp120, including A32 and C11, or to surfaces of gp41 exposed on the postfusion conformation of the protein, only directed ADCC against cells infected with laboratory-adapted HIV-1_{NL4-3}, which is particularly sensitive to antibodies, many broadly neutralizing antibodies (bnAbs) also had excellent ADCC activity against primary virus isolates expressing neutralization-resistant Env. bnAbs with potent ADCC targeted Env epitopes in the CD4 binding site, V2 apex and V3 region of HIV-1 gp120. Furthermore, ADCC activity correlated with binding to Env on the surface of virus-infected cells and with the neutralization of viral infectivity. These results expand the specificities of antibodies capable of directing ADCC against HIV-1 infected cells and suggest that contrary to earlier reports, non-neutralizing antibodies may be ineffective mediators of ADCC against cells productively infected with primary HIV-1 field isolates.

Mechanisms of HIV-1 Resistance to ADCC

ADCC responses have been carefully characterized in the past, and the role of epitope specific neutralizing and non-neutralizing mAb that can recognize HIV-1 envelope at the time of virus entry and budding has been discussed.¹⁴⁻¹⁶ The importance of ADCC responses in the context of natural infection and vaccine preclinical and clinical trials has been widely documented.¹⁶ Nonetheless, HIV-1 has evolved sophisticated mechanisms of immune evasion that enable the virus to replicate continuously in the face of host immune responses. Recent studies have revealed functional activities of HIV-1 accessory proteins, as well as structural features of the viral Env that reduce the susceptibility of virus-infected cells to elimination by antibodies and ADCC in particular. By downmodulating and degrading tetherin (BST-2 or CD317), an interferon-inducible transmembrane protein that inhibits

virus release from infected cells, HIV-1 Vpu prevents the accumulation of nascent virions on the surface of infected cells and reduces their sensitivity to opsonization and killing by Env-specific antibody mediated mechanisms.^{17,18} CD4 downmodulation by the viral Nef protein, and to a lesser extent by Vpu, also protects HIV-1-infected cells from Env-specific antibodies by preventing the formation of Env-CD4 complexes and exposure of CD4-inducible epitopes that are normally occluded in the unliganded conformation of the Env trimer.^{3,7} In addition to these activities of Vpu and Nef, trafficking of Env to the plasma membrane before virus assembly is regulated, in part, by a conserved endocytosis motif in the cytoplasmic tail of gp41. Accordingly, disruption of this motif increases Env expression on the cell surface and susceptibility to ADCC.¹⁹ Studies showing a general correlation between virus neutralization and ADCC when specific mAbs are evaluated,^{13,20} and a lack of ADCC activity for non-neutralizing antibodies against primary HIV-1 isolates,¹³ further suggest that many of the structural features of the Env trimer that make it difficult for antibodies to bind to virions and block their infectivity also confer resistance to antibody-dependent killing of HIV-1-infected cells. In addition to mechanisms to control HIV-1 envelope conformation and the level of envelope expression, there is evidence that HIV-1 can directly escape ADCC through the development of mutations within ADCC epitopes.²¹ Along with these virus-related mechanisms of ADCC evasion, chronic HIV-1 infection also induces reductions in natural killer (NK) cell CD16 expression that decrease the capacity of NK cells to mediate ADCC.²² *In vitro* research suggests that this phenotype of reduced CD16 expression might reflect chronic NK cell activation. Indeed, *in vitro* activation of NK cells results in the cleavage of CD16 from NK cells by matrix metalloproteinases.²³⁻²⁵ Thus, HIV-1 has evolved multifaceted and complementary mechanisms to prevent the elimination of virus-infected cells by antibodies, which helps to explain why antibody responses targeting infected cells, like other mechanisms of immunity, ultimately fail to contain virus replication in most HIV-1-infected individuals.

NK Cell Education and Antibody Dependent Functions

It is well established that NK cells can act as ADCC effector cells. Their activity is governed by the integration of signals received from activating and inhibitory cell surface receptors. NK cell functionality is determined by whether they undergo education through inhibitory NK cell receptor (iNKR) interactions with self major histocompatibility complex class I (MHC-I or HLA-I) ligands. Indeed, the ability of NK cells to exhibit degranulation and/or produce cytokines upon activation with Env-pulsed target cells appears to be influenced by the education process.²⁶⁻³⁰ Until recently, however, the role of NK cell education in determining the capacity of NK cells to mediate antibody-dependent cytotoxicity of HIV-1 Env-pulsed target cells has not been investigated. Recently, Dr. Bernard's laboratory has used the GranToxiLux assay²⁶ to determine the frequency of Granzyme B positive gp120-coated CEM.NKr-CCR5 target cells following incubation with PBMC effector cells in the presence of anti-HIV-1 antibody. This assay detected robust ADCC activity, but effector cells from donors carrying or lacking education competent KIR3DL1/HLA-Bw4, KIR2DL1/HLA-

C2, or KIR2DL2-3/HLA-C1 receptor ligand combinations were no different in their capacity to mediate ADCC. These data could represent an insignificant role for educated NK cells in mediating anti-HIV-1 ADCC; alternatively, it could simply reflect the capacity of other education competent inhibitory receptor/ligand combinations to make compensatory contributions to ADCC potential in effector cells from donors lacking KIR3DL1/HLA-Bw4, KIR2DL1/HLA-C2, or KIR2DL2-3/HLA-C1 combinations. Indeed, NK cell education has been shown to occur through numerous receptor/ligand combinations, including KIR3DL1/HLA-Bw4, KIR2DL1/HLA-C2, KIR2DL2-3/HLA-C1, and NKG2A/HLA-E.^{30–33} Along the lines of this rationale, Dr. Parsons has recently utilized fluorescence activated cell sorting to remove educated NK cells (i.e., those educated through KIR/HLA and NKG2A/HLA-E) from the PBMC population (unpublished data). Furthermore, he has employed the whole PBMC and educated NK cell depleted PBMC in cytotoxicity assays against gp120-coated CEM.NKr-CCR5 target cells. These assays reveal an almost complete elimination of detectable ADCC upon removal of educated NK cells. Overall, these observations imply that the capacity for interindividual genetic differences in carriage of education competent inhibitory receptor/ligand combinations to limit the effectiveness of vaccine strategies targeting NK cell mediated ADCC through the induction of ADCC-competent Abs could be reduced by the presence of additional education competent receptor/ligand combinations.

The involvement of educated NK cells expressing inhibitory KIR and/or NKG2A in anti-HIV-1 ADCC responses raises questions regarding the ability of these receptors to dampen ADCC responses following recognition of their ligands on infected target cells. Indeed, Ward *et al.* previously demonstrated that ADCC of HIV-1-infected primary T cells by a cocktail of four mAb could be dampened through iNKR recognizing HLA-C and HLA-E.³⁴ Given, however, that polyclonal antibodies enriched from patient sera samples trigger more robust ADCC than mAb alone or in combination,³⁵ it remains possible that the antibody cocktail used by Ward *et al.*³⁴ induces suboptimal levels of NK cell activation for NK cells to overcome inhibition through inhibitory receptor ligation. Indeed, Gooneratne *et al.* demonstrated educated KIR3DL1⁺ NK cells to at least partially overcome inhibition through KIR3DL1 to mediate antibody-dependent functions, following stimulation with HIV-1 envelope pulsed primary HLA-Bw4⁺ T cells in the presence of plasma from an HIV-1-infected donor, providing evidence that with optimal stimulation NK cells can be liberated from the effects of inhibitory receptor ligation.³⁶ Future research needs to investigate if sufficient antibody-dependent NK cell stimulation to overcome signals through iNKR can be achieved against HIV-1-infected target cells. Achieving such levels of NK cell stimulation is likely to be complicated by the numerous mechanisms used by HIV-1-infected cells to evade ADCC antibody binding.^{15,37}

Mouse Models to Study Fc Effector Function of Anti-HIV-1 Antibodies

Passive administration of antibodies against HIV-1 Env with broad and potent neutralization activity confers sterilizing immunity against viral challenge and provides durable and sustained control of virus replication in preclinical ani-

mal models of HIV infection, as well as in HIV-infected humans.^{38–43} Compared to conventional pharmacologic mediators, whose activity depends on a single function, that is, enzyme inhibition or receptor blocking, antibodies are multifunctional molecules and their antiviral activity results from the synergistic function of the Fab and Fc domains.⁴⁴ Although Fab-mediated interactions are important for antigen recognition and viral neutralization, interactions of the Fc domain with Fc γ receptors (Fc γ R) on effector leukocytes stimulate distinct immunomodulatory pathways that regulate several aspects of innate and adaptive immunity.⁴⁵ A major drawback in the study of Fc γ R effector activity of anti-Env mAbs is the lack of a robust *in vivo* HIV-1 infection model that would faithfully recapitulate the complete virus infection cycle, human effector cells, and Fc γ R diversity to provide useful insights into the precise contribution of Fc γ R-mediated pathways to the *in vivo* antibody activity. To overcome these limitations, several complementary *in vivo* mouse models have been developed and used to assess the role of Fc γ R-mediated interactions in the *in vivo* activity of anti-Env mAbs.^{38,46–48} Studies using these models revealed that the *in vivo* protective activity of anti-Env mAbs depends on Fc-Fc γ R interactions and potent antiviral activity is achieved through pleiotropic Fc effector functions, including viral opsonization and clearance, as well as elimination of HIV-1-infected cells through Fc γ R-expressing effector leukocytes.^{47,49} Enhancement of these effector functions through augmented activation of Fc γ R-mediated pathways could lead to anti-Env mAbs with improved *in vivo* efficacy. Similar findings have also been reported for several antibodies against other viral, bacterial, and fungal pathogens.^{44,50–56} Disruption of Fc-Fc γ R interactions readily results in reduced *in vivo* antibody activity, whereas preferential engagement of activating Fc γ R is associated with improved therapeutic efficacy, highlighting the importance of Fc effector functions in the antibody-mediated protection against infection.

Conclusion

Recent structural work identified the C1-C2 epitope structure recognized by anti-cluster A antibodies such as A32 and C11. This has been pivotal to understand the importance for the envelope trimers to be recognized following the conformational changes induced by binding to the CD4 receptor. Most importantly, this new information is being exploited in the design of novel vaccine immunogens. It is now clear that the potency of these CD4i recognizing antibodies can be counteracted by the ability of the virus to downregulate both CD4 and tetherin. The use of CD4mc to force Env to expose these ADCC-mediating epitopes might become a powerful tool to be utilized in therapy and prevention along with the anti-cluster A antibodies in combination with additional CD4i Abs, such as those recognizing the CoRBs. Additional strategies aimed at counteracting tetherin downregulation by Vpu might further increase the potency of these non-neutralizing antibodies and should be further explored to increase our ability to develop immune therapeutics that can target highly conserved regions of the HIV-1 envelope.

As of now, there is no perfect assay that can recapitulate ADCC responses against all the different aspects of virus entry and budding. However, the field managed to develop more reproducible assays to investigate differences in epitope

recognition of Env, differences in ADCC responses induced by natural infection versus those induced by vaccines, and analyze the breadth of these responses. Additional efforts should build upon these assays without forgetting that each assay offers the possibility to evaluate different aspects of the ADCC response. Finally, it is important to recognize that there are a variety of effector cells that can mediate Fc-mediated effector functions. With regards to NK cell effectors, all NK cells might not be equal in their potential to mediate ADCC. How vaccines might engage NK cells with higher ADCC potential is of considerable interest for the field. The potential influence that KIR and FcR polymorphisms may have on ADCC and NK responses deserves additional efforts. Translating these findings into animal model is necessary to address the importance of these responses *in vivo*. However, while tremendous progress has been made in evaluating human Fc-mediated responses in animal models, additional work is required to fully represent human responses. Addressing these important questions is required to move the field forward and exploit Fc-mediated responses to fight HIV *in vivo*.

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Author Disclosure Statement

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