EXPERT REVIEWS

HIV-specific antibody-dependent cellular cytotoxicity: a novel vaccine modality

Expert Rev. Clin. Immunol. 8(8), 767–774 (2012)

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A safe and effective HIV vaccine has eluded the scientific community for over three decades. With the failure of vaccines based on neutralizing antibody and cytotoxic T cells, researchers are seeking novel approaches. The partially successful RV144 vaccine trial focused scientific interest on binding antibodies, such as those that mediate antibody-dependent cellular cytotoxicity (ADCC). The biological importance of HIV-specific ADCC is strongly suggested by the generation of ADCC-escape HIV variants and passive transfer experiments. Newer assays for HIV-specific ADCC have defined new epitopes other than in the envelope protein. Such ADCC epitopes could be useful in novel HIV vaccine design. Researchers have shown that recombinant viral vectors such as canarypox or adenovirus boosted with recombinant HIV proteins can induce ADCC and lead to partial protection. These significant developments pave the way for trialing ADCC-based technology in novel HIV vaccine studies.

Keywords: ADCC • antibodies • HIV • NK cells • vaccine

Three decades have passed since AIDS first came to the attention of the scientific community. Major advances have been made in diagnosing HIV as the etiological agent, educating people on how it is spread and developing HAART to treat HIV. Approximately 30 million people have died from AIDS and over 33 million people worldwide are currently infected with HIV. Despite global educational efforts to halt the spread of HIV and major philanthropic and political initiatives to make HAART available to millions more people, the number of newly acquired infections is still increasing [1]. A safe and effective HIV vaccine remains as a global health priority and is the best hope for controlling the AIDS pandemic.

Designing and developing an effective AIDS vaccine capable of overcoming HIV's astonishing degree of viral diversity remains one of the biggest challenges of modern-day science. Innovations in vaccine development are needed that can effectively prime both innate and adaptive immunity in people at risk of acquiring HIV. By 2005, over 60 Phase I/II trials of approximately 30 candidate vaccines had been conducted worldwide [2], all without success.

Challenges to HIV vaccine design & previous HIV vaccine strategies

By infecting and depleting CD4 T cells, HIV-1 damages the immune system, contributing to the inability of humans to clear the virus. Furthermore, despite humans developing vigorous humoral and cellular immune responses toward HIV, no cases of spontaneous clearance and cure have been documented, although a single case of apparent eradication of HIV-1 has been reported following stem cell transplant with a graft comprising donor cells containing the CCR5d32 deletion [3].

The inability of humans to spontaneously clear HIV is partly due to the rapid mutagenesis of the genome [4]. Immune evasion is facilitated by serial antibody escape mutants [5] and glycosylation of envelope proteins, which masks critical epitopes [6]. Vaccines eliciting neutralizing antibody have been effective against homologous strains of HIV but do not neutralize diverse field strains of HIV, nor do they prevent HIV infection [7]. Furthermore, achieving and sustaining the required neutralizing antibody titers has not proved feasible, despite some success with macaque passive transfer studies [8].

 10.1586/ECI.12.74 ISSN 1744-666X

HIV can replicate in the presence of strong HIV-specific cell-mediated immunity [9]. Again, this is mediated by mutations in HIV epitopes leading to escape from T-cell recognition [10]. Nef-mediated reduction in MHC class I expression and gene regulation contributes to latent infection [11]. Vaccine development is further complicated by:

- Enormous genetic diversity of the HIV genome;
- Lack of a precise immune correlate of protection from HIV-1;
- An imprecise animal (simian) model to study primary HIV-1 strains;
- The need to generate mucosal immunity to achieve optimal protection.

Live attenuated Nef-deleted SIV vaccines have effectively induced sterilizing immunity in a macaque model [12]. However, some healthy macaques develop AIDS from attenuated SIV strains [13] while some humans infected with *nef*-deleted HIV-1 strains also slowly progress to AIDS [14]. These concerns about reversion to virulence means that live attenuated HIV-1 vaccines are unsuitable [15]. Antibody-based vaccines have only reliably induced neutralizing antibodies against homologous strains of HIV [16]. Completely inactivated SIV and recombinant protein vaccines have had limited efficacy in macaques [17], with poor persistence of immune responses and no cross-reactive humoral immunity to heterologous strains.

Rerks-Ngarm *et al.* [18] succinctly summarized the outcome of Phase III HIV vaccine trials up to 2009, stating: "Previous efficacy trials of HIV vaccines in higher risk populations have not shown an effect on disease acquisition. Bivalent subtype B AIDSVAX B/B gp120 did not protect high-risk men who have sex with men [19] and AIDSVAX B/E did not protect Thai injection-drug users from infection with HIV-1. The Step trial of Merck [20] recombinant adenovirus type 5 (rAd5) HIV-1 vaccine containing subtype B gag, pol and nef in high-risk men who have sex with men was stopped because of futility and possibly higher rates of infection in vaccine recipients." The continued failure of the scientific community to advance the cause of an effective HIV vaccine lead many to consign this quest to the 'too hard basket', which in turn threatened government funding and involvement from commercial partners [21].

The case for optimism: RV144 vaccine trial

Optimism for the development of an effective vaccine against HIV comes from cohorts of individuals who appear to possess immunity from infection, such as some African sex workers who remain HIV-negative despite multiple exposures. Their immune correlate of protection is unknown; however, it has been noted that in such individuals HIV-specific CD8 cytotoxic T lymphocyte (CTL) and CD4 T-helper responses have been detected, and these may afford protection against HIV infection [22,23]. Greater and more recent exposure to HIV seems to correlate with an increased protection from infection [24], pointing to an acquired mechanism of protection. This could be utilized in a vaccine strategy, although it may not evoke long-lasting protection [24]. Natural killer cell activity has been observed to be increased in HIV-1-exposed but uninfected Vietnamese intravascular drug users; antibody-dependent cellular cytotoxicity (ADCC) activity was noted in this cohort [25]. ADCC is an important bridge between the innate and acquired immune systems, wherein antibodies binding to antigen presented on infected cells and recruit natural killer (NK) cells to kill the virus-infected target.

The Thai RV 144 Phase III vaccine trial conducted from 2003 to 2009, using 16,000 volunteers, was the first (albeit partially) successful vaccine and it has breathed new life into this field of research. This trial initially received criticism from many in the scientific community [21] as it was a combination of two previously failed vaccine strategies: priming with recombinant canary pox (ALVAC) and boosting with recombinant gp120 (AIDSVAX B/E). Previously, a planned Phase III clinical trial using ALVAC-HIV vCP1452 was aborted due to poor immunogenicity data in Phase II studies [26,27], while the poor results of Phase III studies using AIDSVAX B/B and B/E are mentioned above. Surprisingly, this study [18] showed a modest 31.2% reduction in the risk of HIV-1 acquisition among vaccinees, compared with placebo vaccines (51 new HIV infections compared with 74, respectively; $p = 0.04$).

This result has sparked a large postvaccine effort to elucidate the correlates of protective immunity, with a view to improved HIV vaccine design. During the trial, vaccine immunogenicity data were published showing binding antibodies to clade B *env* and clade E *env* were detected in 100% and 96% of vaccinees, respectively, while neutralizing antibodies were detected to clades B and E *env* in 98% and 71% of vaccinees, respectively, although only to tier 1; placebo recipients had no antibody responses [28]. Furthermore, ADCC activity against clade B and E *env* was detected in 96 and 84% of vaccinees, respectively, compared with 11 and 7%, respectively, in placebo vaccinees [29]. These data were obtained using ALVAC-HIV (vCP1521) and AIDSVAX B/E in volunteers in Phase I and II trials in the lead up to the RV144 Phase III clinical trial.

RV144 vaccinees almost universally developed gp120 binding antibodies (98.6 vs 0%, $p < 0.001$; GMT^{-1} 31,207 to clade B MN strain and GMT^{-1} 14,558 to clade A/E A244 strain), while a smaller number of vaccinees developed Env-specific CD4 T-cell responses (34.0 cf. 3.6%; $p < 0.001$). CD8 T-cell responses to Gag did not differ between the groups, while neutralizing antibodies were only detected against the vaccine. A major focus of research has been to further characterize these binding antibodies in an effort to understand the correlates of protective immunity.

Binding antibodies

A non-neutralizing binding antibody when combined with a viral antigen can elicit a variety of effector cell-based immune responses: crosslinking, trapping [30], phagocytosis, complement binding [31] and ADCC. The latter is of increasing interest to vaccinologists as ADCC Abs have been shown to stimulate NK cell effector functions and play a role in protecting from and controlling viral infections [32,33]. Very recently, Haynes *et al.*

reported correlation analyses between immune responses and protection in the RV144 trial [34]. The presence of non-neutralizing IgG-binding antibodies to V1/V2 variable loops in HIV envelope correlated with a 43% reduction in HIV infection rate. For volunteers with high V1/V2 responses compared with those with medium- or low-level responses, there was a 71% reduction in HIV infection rate. Serum IgA antibodies to Env increased risk of acquisition and appeared to block a potentially protective role for ADCC antibodies. Thus, non-neutralizing ADCC antibodies are under intense interest as a potentially protective immune response to HIV.

ADCC in HIV & SIV

ADCC is an important early immune response against viral infection. It efficiently co-ordinates between the humoral and innate branches of immunity by utilizing effector cells bearing Fcγ receptors such as NK cells that bind and kill target cells expressing viral antigen via specific antibodies of the IgG isotype. This may well be a promising strategy upon which to develop an effective vaccine against HIV. Several human cohort studies suggest that the ADCC responses correlate with slower progression to AIDS [33,35,36]. Macaque SIV vaccine studies have suggested a role for ADCC in protective immunity [37]. One potential advantage of NK-mediated ADCC is the ability to target virus-infected cells. This may be beneficial given that, following infection, the predominant mode of dissemination of HIV is thought to be cell–cell spread, rather than via cell-free virus [38]. This transmission occurs via virological synapses [39] and may evade circulating neutralizing Abs [40,41], although this is debated [42,43].

Components of HIV-specific ADCC

Effector cells in ADCC responses

NK cells are one of the major effector cells for ADCC in HIV [36,44,45], as evidenced by antibody-mediated HIV-1 inhibition in the presence of NK cells and antibody-mediated expression of activation markers and cytokine release from $CD56^+$ NK cells; this activation is dependent on the presence of IgG [45], indicative of an ADCC mechanism. The findings from Alter *et al.* have demonstrated an association between the NK cell receptor, KIR3DS1, and its ligand, HLA-Bw4, and slower disease progression, suggesting that NK cells play an essential role in the control of HIV-1 disease [46]. This also includes a direct role that is not ADCC mediated [47], although the capacity to harness this in a vaccine strategy is unclear. Innate immune cells are less susceptible to the immune defects caused by HIV, which destroys CD4 T-helper cells. Other cells of the immune system, in particular monocytes, macrophages, neutrophils and eosinophils, can also mediate ADCC [48]. These effector cells (including NK cells) along with IgG are present in mucosal sites (e.g., the gastrointestinal tract and female genital tract), where they can potentially act early during the very initial events of HIV infection, which is principally transmitted across mucosal surfaces [49,50]. Rapid immune response at mucosal sites of HIV entry are likely to be beneficial in HIV vaccine design. The authors have previously shown that HIV peptide-specific degranulation of NK cells is more rapid than peptide-specific CTL responses [44], offering a potential advantage for an ADCC-based vaccine.

ADCC antibodies

Unlike neutralizing antibodies (which target Env), ADCC antibodies have been shown to target a range of viral proteins such as Nef [51], Pol [52] and Vpu [45], as well as Env. Immune escape mutations within epitopes targeted by HIV-specific ADCC responses have been demonstrated, confirming their biological impact on HIV [53]. These mutations were not driven by CTL pressure, while sequencing of flanking regions and comparisons with database sequences indicated that the mutations within the ADCC epitope were not part of the random variability found within HIV-1; furthermore, purified ADCC antibodies did not show neutralization function [53]. As mentioned above, several studies have shown ADCC antibodies are correlated with slower progression of HIV infection [33,35,36]. Such antibodies are often detected in long-term slow progressors and so-called elite controllers (individuals who are able to control HIV viremia without the use of HAART) [35,54]. Research conducted in nonhuman primates showed that impairing the ADCC function of broadly neutralizing antibodies reduces their protective effect [31], while the potency of monomeric neutralizing antibody 2G12 was improved by introducing ADCC-augmenting mutations, suggesting that the ADCC activity of neutralizing antibodies could improve their vaccine potential [55]. On the other hand, augmentation of the Fc-receptor of b12 neutralizing antibody, while improving its *in vitro* ADCC activity, did not translate to enhanced protection *in vivo* [56].

Studies conducted on ADCC activity in cervical lavage fluids of HIV-1 infected women suggests that ADCC antibodies may be produced locally, and the presence of these ADCC antibodies in the cervicovaginal fluids indicates that this form of innate immunity can contribute to mucosal defense against HIV-1 [57]. It has also been shown that ADCC activity in breast milk is associated with reduced transmission to infants [58].

Target of ADCC: epitopes studied so far

Several HIV-specific ADCC epitopes have been identified, some of which are present in many HIV-infected individuals. As mentioned above, they include Nef-specific ADCC epitopes and recently found Pol and Vpu epitopes, as well as Env [53,59,60]. The authors have demonstrated that Vpu epitopes are enriched in long-term slow progressors [61]. The identification of ADCC targeting internal HIV proteins such as Vpu has initiated research to elucidate the mechanism. Improving the presentation of ADCC epitopes by vaccination should rationally enhance protective immunity. The authors studied whole-blood samples from HIV-positive subjects for the ability of ADCC antibodies to specific epitopes to activate NK cells by using flow cytometry and have identified numerous HIV epitopes. Research to elucidate the mechanism of presentation is underway.

Conformational epitopes to whole Env responses have also been elucidated. Conformational V3 loop ADCC epitopes appear to be a major target for ADCC activity, but ADCC epitopes in the V2 domain of gp120 have also been defined [62]. The authors

have also detected strong (conformational) *env*-specific ADCC in an elite controller patient. It seems likely that the presence of ADCC epitopes in a variable region may result in viral variants escaping ADCC responses. Analyzing for viral escape from ADCC to identify a subset of conserved ADCC epitopes would aid in vaccine design.

ADCC: the role of Fc receptors

HIV infection is associated with a number of changes in FcR expression on effector cells that are associated with changes in their ability to respond to the target cells, potentially contributing to a failure in viral clearance and progressive HIV-1 infection. For example, the potency of ADCC responses has been directly correlated with FcγRIIIa expression [63]. In addition, a good correlation was found between IgG1 Fc-region variants that alter FcR binding and *in vitro* functional NK cell and ADCC assays [64]. The Fab domain and the Fc constant domain of IgG and FcγRs present at the membrane of antigen-presenting cells are involved in efficient inhibition of HIV-1 replication by monoclonal and polyclonal non-neutralizing inhibitory IgG [62].

ADCC assays

Historically, most ADCC assays have relied on killing of radiolabeled target cells either infected with HIV or pulsed with whole HIV proteins [65–67]. Apart from using radioactive material, such assays are difficult to standardize, labor intensive and time consuming. Forthal *et al.* described the antibody-dependent cellular viral inhibition assay, revealing that effector cells such as NK cells mediate potent viral inhibition with otherwise non-neutralizing antibody [36,68,69]. The fluorescence-based ADCC (RFADCC) killing assay has also been developed, wherein membrane and intracellular contents of target cell lines are labeled with fluorescent dyes and loss of intracellular fluorescence is measured by flow cytometry [70]. These assays are quantitative, but usually still rely on the use of artificial cell lines labeled with virus/whole protein as target cells and healthy donor PBMCs as effectors. Furthermore, they cannot easily elucidate the target of HIV-specific ADCC.

The authors have developed a flow cytometric assay that measures activation of effector NK cells following engagement by ADCC antibodies [45]. ADCC activity is measured using 200-µl volumes of whole blood from HIV-infected donors after stimulation for 5 h with HIV proteins or overlapping peptides. NK cells are assessed for expression of activation markers such as intracellular IFN-γ and TNF-α, expression of CD107a and degranulation of granzymes. The assay can be effectively performed using stored plasma and donor NK cells. The enhanced green fluorescent protein (EGFP)-ADCC flow cytometric assay uses EGFP, stably expressed in CEM-NKr cells, a NK-resistant human T-lymphoblastoid cell line, known as EGFP-CEM-NKr cells. The EGFP-CEM-NKr stable cell line provides a novel method to measure ADCC activity to HIV-1 gp120 by flow cytometry without prestaining or prelabeling target cells [71].

Recently, an elegant assay was developed using granzyme B-mediated hydrolysis of a cell permeable fluorogenic peptide to identify ADCC activity mediated by NK cells [72]. The assay, however, can only identify ADCC mediated by Env and is not readily able to identify the specific epitope being targeted.

Inducing ADCC via vaccination

Immune correlates of vaccine protection from HIV-1 infection would provide important milestones to guide HIV-1 vaccine development. ADCC was assessed in volunteers participating in an ALVAC-HIV (vCP1521)/AIDSVAX-A/E, B/E gp120 prime–boost vaccine trial in Thailand [29]. There was a significant difference in the magnitude of the ADCC response to both targets between vaccinees and placebo recipients, demonstrating that this HIV vaccine is a potent inducer of ADCC activity.

A Phase I randomized, double-blind, placebo-controlled trial to assess the immunogenicity of a multiclade HIV-1 DNA plasmid vaccine was conducted in 31 HIV-1-negative Ugandans. This showed that ADCC activity could be induced by DNA vaccination also [73]. Another vaccine trial that used the subtype B Ad-HIV recombinant prime/envelope protein boost regimen has also been shown to elicit broad ADCC activity against diverse HIV clades [70]. Researchers have also elicited ADCC by using a heterologous mucosal prime systemic boost immunization regimen [48]. Priming with replicating adenovirus type 5 host range mutant-SIV recombinants, followed by boosting with SIV gp120, elicited antibodies with ADCC activity against SIV (mac251)-infected cells. *In vitro* ADCC activity correlated with *in vivo* reduced acute viremia after a mucosal challenge with pathogenic SIV [74]. In a similar study [75], priming with replicating adenovirus type 5 host range mutant (Ad5hr)-HIV/SIV recombinants and boosting with gp140 envelope protein enhanced acute-phase protection via ADCC against intravenous SHIV(89.6P) challenge while priming and no boosting or boosting with an HIV polypeptide that induced neutralizing antibodies to the CD4 binding site of gp120 did not afford protection.

Summary

The results of the RV144 HIV vaccine trial have provided a small but significant step towards an effective HIV vaccine. The role of non-neutralizing antibodies has assumed greater significance, in particular HIV-specific ADCC. Identifying the most potent ADCC targets, inducing these ADCC antibodies via vaccination, and testing them in animal models are the next steps towards attaining this holy grail of science.

Expert commentary

HIV-specific ADCC has become a new frontier in HIV vaccine research. The RV144 HIV vaccine efficacy trial highlighted the likely importance of binding antibodies, particularly antibodies mediating ADCC. New assays to study epitopes targeted by HIV-specific ADCC and assess the killing effect mediated by ADCC antibodies are advancing this field of research. The antibody responses to specific regions of HIV envelope reported by Haynes *et al.* are a point in case, offering a potential target for an ADCC-based vaccine. Non-envelope targets of ADCC are now also being reported in the scientific literature and may assist in the control of HIV replication. The great genetic diversity of

HIV, particularly within the envelope protein, has thwarted vaccinologists, while genomic mutation and the damage to the adaptive immune response inflicted by HIV have overcome immune response in people infected with HIV. Targeting conserved or non-envelope ADCC epitopes and harnessing the innate immune system (especially Fc-receptor-bearing NK cells mediating ADCC effector functions) have great theoretical advantages. Advancements in assays detecting ADCC mean that vaccines can now be assessed, in a more refined manner, for their ability to induce potent ADCC responses. The scientific community is now well placed to specifically engineer and test improved ADCC-based vaccines for HIV.

Five-year view

The next 5 years will see an increasing number of HIV epitopes recognized as being targeted by ADCC, particularly epitopes within highly conserved parts of HIV. Additionally, scientists will find ways to map conformational ADCC epitopes, especially those detected in elite controller subjects. ADCC epitopes enriched in this rare population would be very attractive vaccine candidates. More research will be conducted into understanding Fc-receptor phenotypes and genetically engineering the Fc-portion of ADCC antibodies to maximize their killing potential. Furthermore, cytokine augmentation of vaccines will be explored to improve the efficacy of ADCC-based HIV vaccines. Recombinant viral vectors containing potent ADCC epitopes will be tested in simian challenge models for their ability to induce ADCC and protect against SHIV infections. ADCC-based technology will be expanded into other infectious diseases such as influenza and HCV.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Key issues

- With the failure of neutralizing antibody and T-cell-based vaccines, novel strategies are required to produce an effective vaccine against HIV-1, such as antibody-dependent cellular cytotoxicity (ADCC).
- ADCC responses were detected in the partially successful RV144 HIV vaccine trial, which correlated with reduced risk of infection.
- New techniques are available to study ADCC *in vitro* and *in vivo*.
- ADCC targets have been identified in non-env proteins, such as Vpu and Pol.
- ADCC responses force immune escape.
- Identifying ADCC epitopes in people with slowly progressive HIV or elite controllers is possible and may inform rational vaccine design.
- Isolating HIV-specific ADCC antibodies for testing in a simian passive immunization model is required.
- Generating HIV-specific ADCC targeting highly significant ADCC epitopes, likely using priming with recombinant viral vectors and boosting with recombinant HIV proteins, is possible.

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