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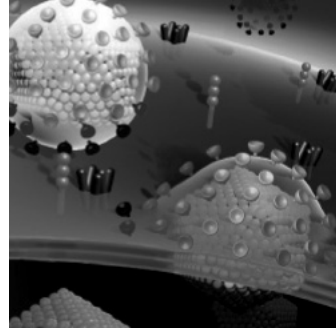

Designing immunity to HIV: manipulating antibody-dependent cellular cytotoxicity antibodies

The failure of HIV vaccine concepts based upon either simple antibody or T-cell immunity means that newer concepts in immunity to HIV require urgent investigation. Antibody-dependent cellular cytotoxicity (ADCC) responses, utilizing the arms of humoral and innate immunity, have been studied for many years, but their role in controlling or preventing HIV-1 remains controversial. Newer technologies can now be applied to study and map ADCC responses. This permits experiments to purify and isolate HIV-specific ADCC antibodies and directly assess their role in preventing simian–HIV infections in macaques. Analogous to complexities in the quality and specificity of T-cell and neutralizing antibody immunity to HIV, it is likely that some ADCC antibodies will be more efficient than others in controlling HIV infection and limiting viral escape. Rationally defining broadly reactive ADCC antibodies with potent *in vivo* activity should allow the selection of the most appropriate ADCC-inducing vaccine antigens. This pathway towards manipulating ADCC antibodies, which control HIV infection, will ultimately improve our understanding of ADCC against HIV infection and potentially yield new HIV vaccine candidates.

KEYWORDS: ADCC ▪ AIDS ▪ antibody-dependent cellular cytotoxicity ▪ chromatography ▪ HIV ▪ immunoaffinity purification ▪ monoclonal antibody

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The problem with HIV

There is a clear need for a HIV vaccine. However, a number of features of HIV make the development of a vaccine very challenging.

The genome of HIV-1 is susceptible to genetic recombination and has an error-prone reverse transcriptase enzyme. This leads to the continuous emergence of mutant HIV-1 variants, even within an individual, facilitating escape from the immune system and greatly complicating the development of a vaccine. Classical vaccine approaches, such as whole-inactivated HIV or protein subunit vaccines, which are aimed primarily at inducing neutralizing antibodies (NAbs), failed efficacy standards in human and macaque trials [1,2]. This is likely a consequence of the vast diversity of HIV-1 strains, extensive glycosylation of the envelope protein, immunodominant variable loops that divert the immune response and effective masking of conserved regions. More recently, vaccine approaches that aimed for an induction and preservation of $CD8^+$ T-cell responses by plasmid DNA or live-recombinant vectors expressing relevant antigen(s) have demonstrated that even a head-start for cellular immunity is no guarantee for the preservation of immune responses or a lower viral-load set-point in HIV infection. Indeed, the recent STEP trial demonstrated that although vaccination with recombinant adenoviruses expressing HIV proteins elicited HIV-specific $CD8^+$ T lymphocytes [3], it did not protect against infection or reduce viral-load set-point in those individuals who became infected during follow-up [4]. These disappointing results demand the investigation of newer concepts in immunity to HIV. It is worth noting that the recently announced Thai HIV vaccine-efficacy trial using a Canarypox/protein prime/boost demonstrated a modest (31%) reduction in new infections. Some observers have suggested that this effect could be due to non-neutralizing or binding HIV antibodies [101]. Several newer HIV vaccine strategies are emerging, although most of the concepts are variations on T-cell or antibody-based ideas **(Table 1)**. There have been numerous calls for the development of more innovative HIV vaccines.

The generation of broadly reactive NAbs is an urgent priority for HIV vaccine development. NAbs can utilize mechanisms such as complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) *in vivo* [5]. Where NAbs act to inhibit the attachment of the virus and fusion between the cell and the virus, ADCC and CDC effector mechanisms can kill free virions

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Table 1. Novel HIV vaccine concepts.

Effector mechanism	Concept	Comment	Ref.
T-cell-based	Novel vectors:		
	Modified vaccinia vectors	Modified to reduce expression of irrelevant Ag targets	[38]
	Simian CMV	CD8-independent effector memory T cells	[39]
	Multiple epitope vaccines:		
	Universal T-cell vaccine	DNA Polytope of 270 CD8 epitopes	[40]
	Polytope 'coldspot' vaccine	Targets recombination 'coldspots' within HIV	[41]
	New administration route:		
	Nasal/vaginal/aerosol delivery	Increased mucosal response	[42–44]
	New augmentation:		
	Electroporative delivery	Enhanced delivery of DNA to cells	[45]
	Cytokine coexpression (e.g., IL-15)	Boosting T-cell immunity	[46]
	Nanoparticles	Protects Ag delivery to dendritic cells	[47]
	Novel host target:		
	LINE-specific T cells	Targets HIV-induced cellular proteins rather than HIV itself	[48]
Neutralizing antibody-based	Reverse immunization	DNA construct to express mAb	[49]
	Immune complexes	Abs complexed to gp120 with adjuvant	[50]
	Self-replicating alphavirus/HIV chimera	Replication stops when immunity neutralizes vaccine	[51]
	Antilipid Abs	Target cardiolipin on host monocytes to block infection	[52]
NK cell-based	NK killing mediated by ADCC	Utilizes NK cells from innate immunity	[17]

Ab: Antibody; ADCC: Antibody-dependent cellular cytotoxicity; Ag: Antigen; CMV: Cytomegalovirus; LINE: Long interspersed nuclear element; mAb: Monoclonal antibody; NK: Natural killer.
Adapted from [53].

and virus-infected cells. Importantly, Hessel and colleagues demonstrated significant decreases in the efficacy of mutated NAbs so that they can no longer elicit ADCC functions, although mutations to eliminate CDC function did not impair their *in vivo* efficacy [6].

Antibody-dependent cellular cytotoxicity & HIV

Antibody-dependent cellular cytotoxicity is an immune response that combines components of innate and humoral immunity. Cells that can be activated by ADCC to provide effector functions via their Fc receptors include natural killer (NK) cells, neutrophils and macrophages. ADCC utilizes effector cells bearing Fcγ (FcγR) or CD16 receptors, such as NK cells attracted by antibodies of the IgG isotype to the target antigens on the surface of virus-infected cells. NK cells are argued to be one of the major effector cells for ADCC in HIV infection, with the roles of neutrophils and macrophages in reducing ADCC effectivity currently not fully defined [7]. NK cells comprise 15% of the peripheral blood lymphocytes. Lysis of virus-infected cells is carried out once the ADCC antibodies are coated onto the surface of target cells and interact with the FcγR of NK cells, eliciting the release of perforin, granzymes and cytokines, including IFN-γ, IL-1, TNF-α and granulocyte macrophage colony-stimulating factor. A series of *in vitro* studies have demonstrated the presence of ADCC antibodies against HIV in the plasma of the majority of subjects infected with HIV-1 [8,9]. Furthermore, Banks and colleagues demonstrated that ADCC can protect peripheral blood lymphocytes from HIV infection in the absence of NAbs or virus-specific cytotoxic T lymphocytes (CTLs) [10]. Since ADCC antibodies can kill HIV-infected cells, in comparison to NAbs that clear free virus circulating in the blood, ADCC could be more efficient in preventing cell–cell spread of the virus [11].

Despite the expanding knowledge of the potential of ADCC antibodies, little is known regarding the specific HIV-1 epitopes that stimulate ADCC. To date, only *Env*- [12] and *Nef*-specific [13] ADCC epitopes have been well characterized in HIV-1-infected subjects, initially mapped by ELISA using overlapping peptides. Progress in elucidating further ADCC epitopes has been slow, in part owing to the inefficient and complex nature of the historical assays for assessing ADCC responses **(Table 2)**. Typical killing-based ADCC assays measured responses to large proteins; however, this assay is not suited to assessing specific responses to smaller peptides in order to map ADCC responses [14,15]. A novel intracellular cytokine staining ADCC method has recently been developed that allows the fine mapping

Table 2. Antibody-dependent cellular cytotoxicity assays.

Assay	Antigen	Advantages in determining the utility of ADCC antibodies
ADCVI assay	Infectious HIV	Directly measures inhibition of viral replication
Fluorometric ADCC assay	HIV proteins	Directly measures killing at a per cell level Simpler than ADCVI assay
Intracellular cytokine staining ADCC assay	HIV peptides	Readily maps ADCC epitopes to individual peptide Measures multiple effector functions of NK cells

ADCC: Antibody-dependent cellular cytotoxicity; ADCVI: Antibody-dependent cell-mediated virus inhibition; NK: Natural killer.

of linear ADCC epitopes [16]. Compared with existing killing-based ADCC assays, no artificial cell line is required, and either the patient's or a healthy donor's NK cells, as the effector cells, can be tested for ADCC activity along with the assessment of NK cytokine production, chemokine production and the loss of perforin or granzymes [17]. The ability to map linear ADCC epitopes permits the identification of the particular ADCC antibody, which can then be studied for its potential to prevent HIV infection, or at least retard the disease.

Isolating pure ADCC antibodies

Monoclonal antibodies (mAbs) are proving increasingly useful adjuncts to therapy for a number of diseases, such as cancer and autoimmunity [18], and they may also prove useful for treating HIV infection. A comparison of ADCC antibodies to NAbs within the sera of HIV-infected individuals has highlighted no significant correlation between the two types of antibodies [19]. However, ADCC antibodies expand earlier following seroconversion compared with NAbs [20]. This early development of ADCC antibodies leads to the anticipation that isolation of these antibodies and their development into a vaccine may be the 'needle in the haystack', which sews together the patches of many years of research. Since the development of methods to map specific HIV ADCC epitopes, purification of their antibodies from serum is promising to allow the assessment of their utility. Antibodies have been purified from patient sera with only antibodies of the IgG isotype having been shown to mediate ADCC [21]. The more efficient IgG isotypes IgG1 and IgG3, but also to some extent IgG2 and other mAbs, in mouse sera have also been shown to mediate ADCC [22].

Immunoaffinity chromatography

Antibodies are generally purified from serum or tissue culture supernatants using conventional methods of column chromatography **(Table 3)**. In respect to ADCC antibodies, the optimal technique for purification is most likely immunoaffinity purification **(Figure 1A)**. This method involves the covalent binding of pure antigen to a solid support, allowing the antibodies from the polyclonal sera to bind, first removing the unbound antibodies by a number of wash steps and then finally eluting the antibodies of interest. The strength of this method is to isolate the specific antibodies from a mixed polyclonal pool of sera. However, a disadvantage is that it requires large amounts of antigen and the elution conditions of the specific antibody may lead to some loss of the antibody activity. This technique of purification may also

Table 3. Antibody-dependent cellular cytotoxicity antibody purification techniques.

Technique	Source of antibody	Uses	Advantages	Disadvantages
Protein A beads	All sources	IgG that binds protein A	▪ Easy ▪ Single step ▪ High yield	▪ Expensive ▪ Not suitable for all species and classes
Antigen affinity column	Sera	Antigen-specific antibodies	▪ Yields pure antibodies	▪ Expensive ▪ Multiple steps ▪ Requires pure antigen ▪ Can inactivate antibodies
Anti-Ig affinity column	Sera	Rat, sheep, goat antibodies Class- and subclass-specific antibodies Rat monoclonal antibodies	▪ Yields species- and class-specific antibodies	▪ Expensive ▪ Low-to-moderate yield ▪ Multiple steps

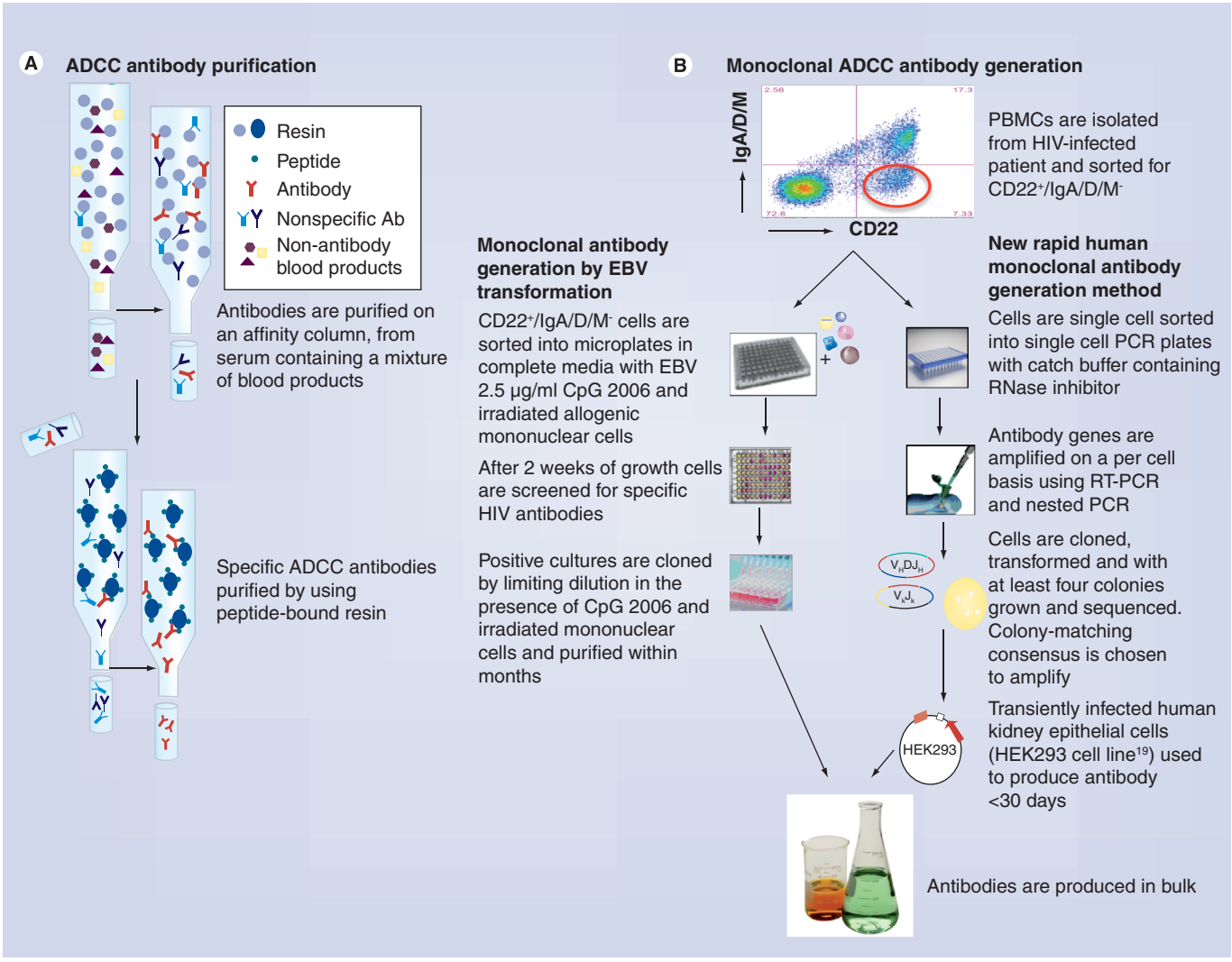

Figure 1. Methods to purify and clone ADCC anitbodies.
Ab: Antibody; ADCC: Antibody-dependent cellular cytotoxicity; PBMC: Peripheral blood mononuclear cell; RT: Real-time.

be further optimized by the use of activated beads, such as cyanogen bromide-activated agarose or coupling peptides to a protein carrier, which is then bound to the activated beads. This technique is efficient for high-affinity antibodies; however, if there is a low affinity between antigen–antibody binding this may hinder the amount of antibody binding to the antigen in the first place. An additional factor that influences the yield of purified antibody is the relative ease with which the antibody can be eluted; this will depend on the type and number of bonds that form at the antigen–antibody interaction site and can be manipulated by changing variables, such as pH. Purification of the specific ADCC antibodies by adapting these techniques will allow consideration of the biological utility of these antibodies and further their characterization.

Monoclonal ADCC antibody generation

An alternative approach to studying ADCC responses is to generate mAbs that recognize specific HIV epitopes. The usual techniques for generating mAbs have a disadvantage in that many of the mAbs generated will not retain robust ADCC activity, since the human Fc portion of the antibody largely governs this specificity. Epstein–Barr virus (EBV)-transformed B cells normally produce considerable amounts of antibodies, but a disadvantage of bulk transformation of peripheral B cells is that it results in populous, polyclonal antibodies. However, Lanzavecchia and colleagues have recently optimized the technique to generate human mAbs by EBV transformation of sorted peripheral blood memory B cells [23]. This technique has been successful in anti-SARS and anti-H5N1

influenza human mAbs from subjects with prior infection [23–25]. This improved technique adapted to obtaining ADCC mAbs from HIV-infected subjects involves adding EBV, CpG oligonucleotides and irradiated filler peripheral blood mononuclear cells to $CD22^+$/$IgA/D/M^-$ B lymphocytes from HIV-positive subjects and screening growing cells for antibody production. Individual clonal B-cell lines are then obtained by limiting dilution **(Figure 1B)**. The resulting mAbs should retain ADCC activity since they are derived from the subject's own B cells.

More recently, there has been further development in the generation of human mAbs by Wrammert *et al.*, who describe a rapid cloning technique (within 30 days) to generate high-affinity human mAbs against the influenza virus [26]. The technique involves sorting human blood cells from antibody-secreting cells by flow cytometry and distributing these cells, one cell per well, into multiwell plates. PCR amplification of the heavy chain (V_H) and κ isotype of light chain region (V_κ) genes of each cell follows cDNA generation by reverse transcriptase. Finally subcloning and screening for mAb expression against the influenza subtypes completes the method. This technique was recently applied to studying large panels of monoclonal NAbs to HIV by first sorting memory B cells that have IgG on the surface binding fluorescent Env protein. This impressive study uncovered a large breadth of NAb in most subjects [27]. Lewis and colleagues recently found this method of cloning of Ig light and heavy genes to be technically more feasible than EBV transformation of B cells [28]. However, it is worth noting that when applied to ADCC, the Fc portion of the Ig molecules must also be cloned out to recombine into a fully competent ADCC mAb.

Many ADCC mAbs to Env may also have neutralizing activity and this can make it difficult to determine the utility of ADCC alone [29]. An elegant study by Hessell *et al.* demonstrated that removing the monoclonal NAbs capability to bind Fc receptors greatly reduced the ability of this NAb to mediate protection from a genetically engineered hybrid virus having an HIV envelope and an SIV core (SHIV) challenge *in vivo,* which correlated with reduced ADCC *in vivo* [6]. Dissecting the role of ADCC antibodies in control of HIV should be greatly facilitated by the recent development of rapid methods to generate fully human mAbs.

Evaluating 'useful' ADCC antibodies

A constructive debate has emerged in recent years regarding the specificity and functions of anti-HIV $CD8^+$ T cells and how this information should be used to design better HIV vaccines. For example, CTLs that target conserved and functionally important regions of *Gag* are likely to incur larger 'fitness' costs to viral replication if escape mutations are accrued [30]. Furthermore, CTLs that express multiple cytokines and chemokines, and rapidly kill virus-infected cells are also likely to be more inducible by vaccination [31]. There is a growing appreciation that the quality of the antiviral immunity, rather than simply the quantity, will ultimately be more important in determining their efficiency in controlling and preventing HIV. What then are the potential parameters that should be analyzed in assessing the quality of anti-HIV ADCC responses **(Table 4)**?

A major issue for the efficacy of HIV-specific ADCC is that the majority of HIV-specific ADCC studied target the HIV-1 Env protein. Env is highly variable between strains, although the largest differences are concentrated within variable loops. Env-specific ADCC may, unless extremely broad or targeted to some of the relatively conserved regions within Env, have limited utility against divergent HIV strains. Targeting ADCC to more conserved proteins, such as Gag, Pol and some of the regulatory/accessory proteins may, if possible, be a more effective ADCC-based vaccine approach. Indeed, Gag-specific $CD8^+$ CTLs are more effective than Env-specific CTLs in both humans and macaques [32,33] – this may also be true for ADCC responses. One reason why Gag-specific CTL responses are relatively more effective is that immune escape often comes at the cost of significantly reduced viral replication. Indeed, it is likely, although not studied to date, that ADCC responses also force viral escape. HIV-specific ADCC epitopes that target functionally constrained viral sequences could, if they force viral escape, lead to less-fit viral strains and slow replication and disease progression.

Natural killer cells are major effector cells of ADCC antibodies. There is considerable heterogeneity among NK cell subsets and this impacts which subsets are activated by ADCC, and the number and type of effector functions triggered. Furthermore, there is considerable evidence of NK cell dysfunction during HIV infection, which may limit the effectiveness of ADCC responses. By comparison to CTLs [31],

Table 4. Issues around the efficacy of HIV-specific antibody-dependent cellular cytotoxicity.

Issue	Comment	Ref.
Protein targeted	Conserved internal proteins are more antigenically similar across strains than Env protein	[14,32]
Viral escape	Escape at functionally constrained epitopes will inflict a bigger fitness cost on viral replication and slow disease	[30]
Antigen presentation	Some epitopes may be better presented on the surface of HIV-infected cells and result in bigger reductions in viral replication	[54]
FcR binding	Broader use of FcRs by the ADCC Fc portion may activate larger numbers of NK cells	[58]
Poly-functional NK cells	ADCC-activated NK cells with the capability of expressing multiple cytokines/chemokines may be more efficient at reducing viral replication	[31]
Rapidity of killing by ADCC-activated NK cells	Killing of virus-infected cells by ADCC-activated NK cells may most effectively suppress virus replication	[17,34,55]
ADCC epitopes associated with slow HIV progression	Particular ADCC responses may be over-represented in subjects with a slow decline of CD4 T cells or low viral load	[35]
ADCC responses associated with prevention of SIV/HIV infection of macaques	Vaccine studies or passive infusion studies in macaques may reveal the most effective type/epitope of ADCC responses	[6,56,57]

ADCC: Antibody-dependent cellular cytotoxicity; FcR: Fc receptor; NK: Natural killer.

it may be that polyfunctional NK cells (those able to express multiple effector molecules), and NK cells, which can rapidly degranulate to kill HIV-infected cells [17,34], may be the most effective cells to recruit by ADCC antibodies.

Although *in vitro* studies on the effectiveness of HIV-specific ADCC responses will help guide the rational selection of ADCC-inducing vaccine antigens, there are opportunities to assess the potential *in vivo* effectiveness of ADCC responses targeting specific HIV epitopes in carefully planned studies of HIV-infected subjects. We suggest that one approach is for cohorts of antiretroviral therapy-naive subjects to be mapped for epitope-specific ADCC responses [16,17]. Rates of progressive disease (e.g., $CD4^+$ T-cell decline) can then be determined for each epitope. Similar approaches were performed studying the rates of disease for various HLA-restricted CTL epitopes, revealing highly useful CTL epitopes [35].

Definitively assessing the *in vivo* protective capability of different ADCC antibodies can utilize SHIV challenge models in macaques. Individual or combinations of ADCC antibodies can be infused prior to virus exposure and virus levels assessed after exposure [6]. A number of HIV-1 genes have been inserted into SIV backbones (e.g., *Env*, *Tat*, *Rev* and parts of *Pol* [36,37]), although not all strains replicate to high levels or result in immunodeficiency. Theoretically, combinations of ADCC antibodies should offer broader coverage of divergent viral strains and/or limit opportunities for viral escape.

Conclusion

There is an urgent need to explore newer concepts in HIV vaccines. The development of simpler ADCC assays, the ability to map ADCC epitopes along with improved methods of purifying and generating mAbs now permits a more definitive evaluation of this utility of ADCC in protection against HIV infection.

Future perspective

There is a growing interest in harnessing ADCC antibodies and the innate immune system in the quest to develop effective HIV vaccines or immunotherapies. A path has been presented in this article on ways to better define, generate and evaluate the most effective ADCC antibodies. Although purified ADCC antibodies could be of direct utility as immunotherapy or immunoprophylaxis, the greatest public-health benefit from the study of HIV-specific ADCC could come from the ability to incorporate the most useful ADCC epitopes into novel preventative HIV vaccines.

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Executive summary

Potential benefits of antibody-dependent cellular cytotoxicity antibodies

- Antibody-dependent cellular cytotoxicity (ADCC) antibodies are part of the innate immune response, hence a possible vaccine can aid the immune system to act rapidly against HIV infection.
- ADCC antibodies could potentially act against a broad range of divergent viral strains and also limit viral escape.

Generating & purifying ADCC antibodies

- Antibodies can be purified from neat serum using immunoaffinity columns.
- High-affinity ADCC monoclonal antibodies can now be rapidly generated by newer methods, which utilize Epstein–Barr virus-transformation or direct PCR.

Conclusion

- With the development of new technologies that identify and map ADCC responses, along with the improvement of purification techniques, novel HIV-preventative vaccines can be designed to make use of this potentially powerful immune response.

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