The Utility of ADCC Responses in HIV Infection

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Abstract: Simple antibodies or vector-induced T cell immunity are unable to provide broad immunity to HIV. Although broadly reactive neutralising antibodies are a goal of vaccination, this remains elusive. There is growing evidence that HIV-specific antibodies that mediate their activity *via* the Fc-receptor, such as antibody dependent cellular cytotoxicity (ADCC), have an important role in controlling HIV infection. Newer assays are being developed that enable HIV-specific ADCC responses to be finely mapped. In turn, this should allow a more definitive analysis of the effectiveness of HIV-specific ADCC antibodies. However, progressive dysfunction of effector cells that mediate ADCC responses, such as NK cells, combined with immune escape variants that emerge from effective ADCC responses, likely undermine the utility of ADCC responses during chronic HIV infection. Nonetheless the utility of ADCC responses in preventing HIV infection requires urgent consideration.

Keywords: HIV, antibodies, ADCC, vaccine.

Renewed interest surrounds non-neutralizing antibodies against HIV as potential vaccine or therapeutic targets. The continued failure to identify immunogens that reliably induce broadly neutralizing antibodies or induce sufficiently broad and powerful T cell immunity to control HIV infection has led to calls for lateral thinking in the generation of effective immune responses [1-3]. Antibody dependent cellular cytotoxicity (ADCC) has been studied for over 20 years in HIV infected individuals, but the interest in this type of immune response has waned in recent years. There are less than 50 publications listed in PubMed searches of HIV specific ADCC compared to hundreds or thousands of articles related to HIV specific T cells or neutralising antibodies. In part the difficulties in studying ADCC reflect the cumbersome nature of the assays typically used. Most assays use radiolabelled target cells or require *in vitro* cell culture techniques [4]. The advent of IFNγ expression type assays such as Elispot and intracellular cytokine staining has revolutionised assays of HIV specific T cell immunity. Similarly, the advent of simpler pseudotyped based neutralising antibody assays has also revolutionised that field. Herein we describe how new assays are being used to potentially harness HIV specific ADCC responses in the induction of protective immunity.

ADCC ASSAYS

Until recently most ADCC assays have relied on the killing of radiolabelled target cells either infected with HIV or pulsed with whole HIV proteins (Fig. **1**). The effector cells are typically healthy donor PBMCs and antibodies are derived from the serum of HIV infected individuals. Most cell lines have been T cell lines such as CEM cells. The readout of these assays is the proportion of cells killed as evidenced by release of intracellular ^{51}Chromium. These assays are notoriously difficult to standardise and are time consuming and involve significant radioactivity.

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More recently, antibody-dependent cellular viral inhibition (ADCVI) assays have been described and utilised in the study of S/HIV-specific ADCC, revealing that effector cells such as NK cells, monocytes and possibly neutrophils mediate potent viral inhibition with otherwise non-neutralising antibody or enhance the biological activity of neutralising antibody [4, 5]. Activity mediated through antibody (Ab) binding to the Fc receptor (as needed by ADCC) is required for effector activity in this assay.

Fluorescence based ADCC killing assays have also been studied with success in recent years [6]. This involves labelling the membrane and intracellular contents of target cell lines with fluorescent dyes and using a flow cytometry approach to look for the loss of intracellular fluorescence. These assays are much more quantitative than Chromium release assays, however usually still rely on the use of artificial cell lines labelled with virus/whole protein as target cells and healthy donor PBMCs as the effectors.

We recently described a flow cytometric assay that measures the activation of effector cells such as NK cells following engagement by ADCC Ab [7] (Fig. **1**). In its simplest form, small volumes (200μl) of whole blood from HIV-infected subjects are stimulated for 5-6hr with whole HIV proteins or overlapping linear 15-mer peptides, and $CD56^+$ $CD3^-$ NK lymphocytes (a component of the innate immune system) are assessed for expressing intracellular IFNγ (Fig. **1**). The activity is mediated by IgG antibodies in plasma and the assay can be performed on stored plasma samples using donor NK cells. The ability of the HIV-specific ADCC antibodies to trigger multiple functions of NK cells (e.g. express IFNγ, TNFα, degranulation of granzymes etc) is also readily monitored in this assay since the NK cells can be accurately gated upon. We have summarized the advantages and disadvantages of the different ADCC assays (Table **1**).

EFFECTOR CELLS IN ADCC RESPONSES

Several cells of the innate immune system can be triggered by ADCC antibodies to provide effector functions mediated by ADCC. NK cells [8], neutrophils and macrophages

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Table 1. Comparison of ADCC Assays

Assay	Advantages	Disadvantages	Reference Example
^{51}Cr release assay (CRA)	1. Directly Measures killing	1. Labour intensive 2. Need cell line targets 3. Uses radioactivity 4. High background 5. Semi-quantitative 6. Cannot phenotype 7. Time consuming 8. Must isolate PBMCs 9. Difficult to map epitopes 10. Not used easily for high throughput	[44]
Antibody Dependent Cell-mediated Virus Inhibition (AD-CVI) assay	1. Measures inhibition of viral replication 2. Can phenotype response	1. Need cell line targets 2. Time consuming (5-7 days) 3. Must isolate PBMCs 4. Does not map epitopes 5. Does not measure direct killing 6. Not used easily for high throughput	[4]
Fluorometric antbody-dependent cellualar cytotoxicity (RFADCC) assay	1. Directly measures killing at per cell level 2. Can phenotype response 3. Short (4h) incubation	1. Requires whole protein 2. Needs fluorescently labelled target cell-line 3. Must isolate PBMCs 4. Does not map epitopes 5. Not used easily for high throughput	[6]
Intracellular Cytokine Staining ADCC (ICS-ADCC) assay	1. Performed on whole blood 2. Short (5h) incubation 3. Can phenotype response 4. Readily maps epitopes 5. Lower background 6. Measures multiple effector functions	1. Does not measure direct killing 2. Not used easily for high throughput	[7]

[9] all have Fc receptors that can be engaged by ADCC antibodies. As cells of the innate immune system, an inherent potential advantage of ADCC responses is their ability to rapidly trigger effector functions. Large numbers of these cells are present at all times in peripheral tissues, in comparison to much smaller numbers of memory B and T cells resident in lymphoid tissues, which require activation for neutralising antibody or T cell effector functions. Since HIV infection rapidly spreads during the first 2 weeks after exposure, a significant time advantage may facilitate much better control of viremia.

Natural killer (NK) cells have been suggested to be one of the major effector cells for ADCC in HIV [10]. Furthermore, the literature describe IgG1 and IgG3 as the most common IgG isotype to mediate ADCC via strong interactions with the Fc-binding receptor CD16/FcγRIIIα, expressed preferentially on NK cells [11]. Along with degranulation of perforin and granzymes upon crosslinking of Fcγ receptors by anti-CD16 antibody, NK cells also secrete several cytokines, such as IFNγ and TNFα, and β-chemokines, which have been shown to inhibit HIV replication *in vitro* [12].

Several studies have now described dysfunctional NK cell activity within HIV infected patients (reviewed in [13]). These defective responses included a lack of responsiveness to cytokines [14, 15], changes in expression of cell surface markers including the selective loss of cytolytic CD16hiCD56low NK cells, the proliferation of a defective CD16hiCD56- NK cell subset [14, 16], and defective ADCC activity *in vitro* [17, 18]. These defects are at least partially reversed after successful antiretroviral therapy [19]. It is plausible that dysfunction of NK cells in the setting of chronic untreated HIV infection results in a waning of the *in vivo* effectiveness of ADCC responses, which may ultimately contribute to the inability of hosts to control HIV infection. Dysfunction of other ADCC effector cells, such as macrophages and neutrophils, has also been described in HIV infected subjects [20-22]. The role of dysfunction of these cell types in reducing the effectiveness of ADCC antibodies is not known.

HIV-SPECIFIC ADCC EPITOPES

Very few ADCC epitopes have been described in subjects infected with HIV. In part this reflects difficulties in mapping epitopes with past standard assays. Previously, this has been achieved by using lengthy cell culture techniques with immortalised B cells from HIV-infected individuals to produce panels of monoclonal antibodies. These are then screened for ADCC activity, using whole protein (usually HIV Envelope, a common ADCC target). The exact epitope is then defined using an ELISA technique and peptides li-

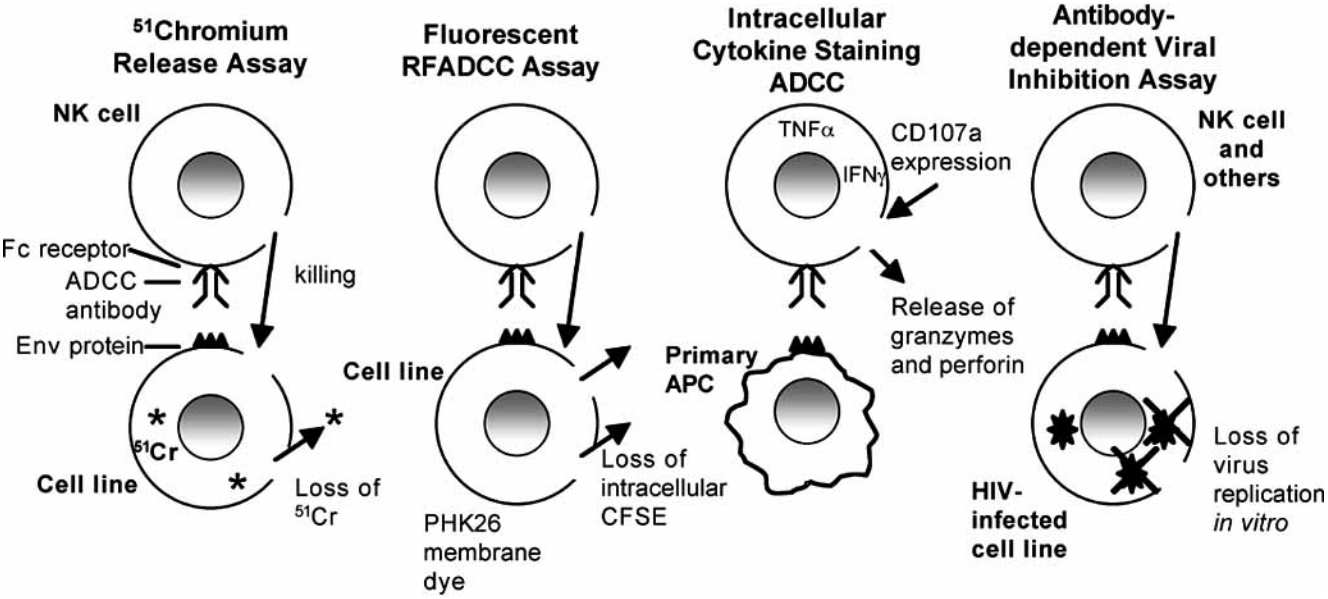

Fig. (1). Schematic comparison of different ADCC assays.

braries [23]. A single Nef-specific ADCC epitope has been described wherein a pool of Nef peptides that was screened for antibody binding using ELISA, followed by the generation of a rabbit monoclonal antibody. This rabbit mAb was then tested for ADCC activity using a ^{51}Cr release assay [24, 25].

With our recently described ICS-based ADCC assay, we made the surprising observation that ADCC could be triggered by linear overlapping HIV peptides [26]. How the peptides are presented to the ADCC antibodies is not yet known, but this results in a ready ability to map specific linear ADCC responses to individual peptides within a pool, much as has been performed to map linear T cell epitopes. We have recently defined several ADCC epitopes across HIV proteins, some of which are shared by multiple HIV infected individuals. A disadvantage of using linear peptides is that only linear antibody epitopes are defined which probably represents a minor fraction of the many conformational antibodies present. Further, for variable proteins such as HIV-1 Env, many ADCC responses in subjects will be missed using a consensus set of peptides – this problem is also true for the use of single strains of Env proteins. Studies of T cell immunity using expanded peptide sets covering larger numbers of strains have identified larger numbers of T cell epitopes, and the same principle is likely to be true for ADCC responses [27]. Nonetheless, the ability to rapidly define even this subset of ADCC antibodies is a significant advance in studying these responses.

EVIDENCE THAT ADCC RESPONSES CONTROL HIV INFECTION

The preponderance of published evidence suggests a role for ADCC in controlling HIV replication in humans or SIV replication in macaques, summarised in Table **2**. A number of studies over the last 20 years have attempted to correlate levels of ADCC antibodies with control of HIV infection. Several cross sectional cohorts of HIV infected individuals

Table 2. Examples of Effectiveness of ADCC Response in Controlling HIV/SIV

Study Type [References]	Cohort	Results	Limitations	Other References
Positive Cohort Study [28]	118 HIV-1 positive men.	Low ADCC titres correlate with progression and CD4+ cell decline.	ADCC titres not correlated to viral load.	[29, 30, 44-46]
Negative Cohort Study [32]	Haemophilia patients (n=13) infected by a common source	No significant difference in ADCC values between those who remain asymptomatic and those who progress to disease.	Very small cohort.	[31]
Monkey Vaccine Study [35]	47 Rhesus Macaques	Vaccine elicited ADCC activity to gp120 protein correlated with reduced viral loads	Vaccines induced other immune responses that may have contributed to efficacy	[47]
Monkey Passive Transfer Study [5]	Rhesus Macaques infused with NAb variants and challenged with $SHIV_{SF162P3}$	Abrogating Fc binding substantially reduced efficacy of NAb. No effect on efficacy of reducing complement.	Used efficient NAb that is rare in infected subjects. Whether non-neutralizing ADCC also similarly effective not yet studied.	
Macaque Cohort Study [48]	5 rhesus macaques infected with SIV_{17E-Br}	Animals with higher ADCC responses progressed more slowly.	Small cohort. Other immune responses may have contributed to slow disease	[49]

demonstrate an association between strong ADCC responses and slower progression of HIV disease or lower viral loads and higher CD4 counts [28-30]. In contrast, other similarly designed smaller studies have not shown a strong association between ADCC responses and slower HIV progression [31, 32]. ADCC can inhibit HIV by inducing the lysis of virus-infected cells before they can release mature virions. However, other investigators have speculated that ADCC may accelerate the progression of HIV by killing uninfected CD4+ cells that have passively bound gp120 [33].

Some of the difficulties reconciling these divergent studies may be explained by different methodologies used or the insensitivity of killing-based ADCC assays. It is also likely that ADCC responses to only a subset of epitopes are associated with control of HIV viremia and prevention of progression, while other epitope-specific ADCC responses may be unhelpful. For CD8 T cell immunity the ability to map responses across large numbers of subjects has shown that the targeting of conserved segments of proteins, such as those with p24 Gag, is much more successful at controlling HIV than other T cells with other specificities [34]. ADCC responses to conserved protein fragments within HIV may be similarly successful but, unlike T cell responses, not restricted to subjects with specific MHC alleles. Large scale mapping of HIV-specific ADCC responses may be required to identify the most useful ADCC epitopes.

EFFECTIVENESS OF HIV VACCINE-INDUCED ADCC RESPONSES

The ability to define the effectiveness of ADCC responses has been hampered both by (a) technical limitations and (b) the problem that vaccines comprising or expressing whole HIV proteins (particularly Env) may stimulate a variety of responses in addition to ADCC. Recently, however, Gomez-Roman *et al*. have demonstrated that ADCC activity against SIV infection can be induced within rhesus macaques by prime/boost AIDS vaccination and that these responses correlated with subsequent control of infection [35]. HIV-specific ADCC responses have been induced in human recipients of a recombinant Canarypox vaccine and Env protein prime/boost regimen in Thailand [36].

Passive transfer studies have the potential to define the protective role of ADCC antibodies. Hessell and colleagues recently showed that the passive transfer of a neutralizing antibody to HIV-1 Env was much more effective in controlling SHIV challenge in macaques if it was able to bind to Fc receptors, implying that ADCC activity may assist in control of viremia [5]. The ability to map, isolate and passively transfer specific HIV-specific ADCC antibodies (without neutralization activity) would greatly assist efforts to define the utility of HIV-specific ADCC antibodies.

Importantly, antibodies that can mediate ADCC against HIV are also present in cervicovaginal fluids of HIV infected women, which indicates that ADCC can potentially contribute to mucosal defense against HIV [30, 37]. The primary effector cells of ADCC (NK cells, neutrophils and macrophages) are present in key sites of HIV replication suggesting they could help mediate control of viral replication [22, 38].

IMMUNE ESCAPE FROM ADCC

HIV evolves at extremely high rates due to its low fidelity RNA polymerase and high replication efficiency [39]. The rapid evolution of HIV is influenced by focused immune responses directed at particular antibody or T cell epitopes (reviewed [40]). These targeted immune responses exert significant immune pressure upon these epitopes, resulting in the rapid appearance of escape mutations [41]. The rate of viral escape likely reflects the strength of the immune pressure and the fitness cost of the mutant virus [42]. The efficacy of particular Gag-specific T cell responses likely reflects high fitness deficits incurred if mutational escape occurs. No previous studies have yet definitively demonstrated viral escape from ADCC responses, although this is likely. Evaluating viral escape from particular ADCC responses should identify a subset of ADCC epitopes that are difficult to escape from or incur significant fitness costs when escape occurs, much as is being utilised in T cell based vaccine design [43]. Such epitopes could be valuable vaccine antigens.

CONCLUSIONS

The protective ability of ADCC antibodies has been largely overlooked in HIV vaccine development in the past probably in part because it has been more difficult to study in comparison to T cell and neutralising antibody responses. However, with the development of simpler assays and an ability to map ADCC epitopes, several new avenues will be opened for research in this field.

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