Antibody Functional Assays as Measures of Fc Receptor-Mediated Immunity to HIV - New Technologies and their Impact on the HIV Vaccine Field

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Abstract: Background: There is now intense interest in the role of HIV-specific antibodies and the engagement of FcγR functions in the control and prevention of HIV infection. The analyses of the RV144 vaccine trial, natural progression cohorts, and macaque models all point to a role for Fc-dependent effector functions, such as cytotoxicity (ADCC) or phagocytosis (ADCP), in the control of HIV. However, reliable assays that can be reproducibly used across different laboratories to measure Fc-dependent functions, such as antibody dependent cellular cytotoxicity (ADCC) are limited.

Method: This brief review highlights the importance of Fc properties for immunity to HIV, particularly FcγR diversity and function. We discuss assays used to study FcR mediated functions of HIV-specific Ab, including our recently developed novel cell-free ELISA using homo-dimeric FcγR ectodomains to detect functionally relevant viral antigen-specific antibodies.

Results: The binding of these dimeric FcγR ectodomains, to closely spaced pairs of IgG Fc, mimics the engagement and cross-linking of Fc receptors by IgG opsonized virions or infected cells as the essential prerequisite to the induction of Ab-dependent effector functions. The dimeric FcγR ELISA reliably correlates with ADCC in patient responses to influenza. The assay is amenable to high throughput and could be standardized across laboratories.

Conclusion: We propose the assay has broader implications for the evaluation of the quality of antibody responses in viral infections and for the rapid evaluation of responses in vaccine development campaigns for HIV and other viral infections.

Keywords: HIV, antibodies, Ig, Fc receptors, natural killer cells, ADCC, ADCP, vaccine.

1. INTRODUCTION

Fc-gamma receptors (FcγR) link the innate and humoral arms of the immune system by engaging IgG antibodies (Ab) and so enabling leukocyte antibody effector functions. Abs bind via their F\textsubscript{ab} region to viral epitopes and their Fc portions bind and cross-link Fc-gamma receptors (FcγR) on effector cells such as NK cells, monocytes, macrophages and neutrophils [1-3].

The FcγRs bind IgG but vary in their tissue distribution and expression on innate cells and functional properties. FcγRs are key components of both IgG-induced protective inflammatory processes and the regulation of immune responses [1-4]. There is increasing recognition that IgG1 and IgG3 interactions with FcγRs are important in immune defence against viral infections [2, 5]. Indeed FcγRs underlie many effector functions of anti-HIV-1 antibodies, both neutralising antibodies (NAb) and non-neutralising Ab, including ADCC [6], antibody dependent cellular phagocytosis (ADCP), antibody dependent cellular viral inhibition (ADCVI) and antigen presentation [7-10]. While both non-NAbs and NAbs engage FcγR mediated effector functions, neutralising antibodies to Env occur relatively late in infection and bNAb may take years to develop [11, 12].

The human and non-human primate (NHP, including macaque) FcγR family (Fig. 1) consists of several activating receptors (FcγRI, FcγRIIA, FcγRIIIa) and a single inhibitory receptor, FcγRIIB. Some human leukocytes, such as macrophages, can express all the FcγRs: activating FcγRI, FcγRIIA, (FcγRIIc), FcγRIIIa, and inhibitory FcγRIIb; thus, the cellular response to IgG-opsonised targets is potentially complex resulting from either the integration of signals from multiple FcRs or the dominance of one receptor signal over the oth-
ers. Different FcγR receptors expressed on different immune cells act different anti-viral immune effector mechanisms [13-17]. Importantly, there is evidence that each activating FcγR can contribute to some protective effects of anti-HIV antibodies (e.g. [18-23]). Aggregation of cellular Fc receptors [24], or possibly their reorganisation in the membrane [25] leads to the activation of src family and syk kinases which initiate a cell activation cascade and, depending on the innate cell type, stimulate effector functions such as ADCC and ADCP [1-3, 26].

Further complexity in Fc-function arises from differences in the specificity of human FcγRs and their affinities for IgG subclasses [27, 28] and can be further affected by genetic polymorphisms of the receptors (Table 1) [26, 29]. FcR categorised as high-affinity bind uncomplexed, monomeric Ab. In the case of FcγRs categorised as low-affinity, binding of monomeric IgG to cells expressing these receptors is not normally detectable while complexes of IgG are strongly bound. For the low affinity receptors FcγRIIa and FcγRIIIa, reactivity broadly follows the hierarchy IgG3 > IgG1~ IgG2 > IgG4 [1-3, 27]. Thus effective ligand binding by these lower-affinity cellular receptors is not normally detectable while complexes of IgG are strongly bound. For the low affinity receptors FcγRIIa and FcγRIIIa, reactivity broadly follows the hierarchy IgG3 >> IgG1~ IgG2=IgG4, except for the allelic variants of FcγRIIa with a Histidine at position 131 (H131) which displays strong binding to IgG2 and has the binding hierarchy, IgG3 > IgG1~ IgG2 > IgG4 [1-3, 27]. Thus effective ligand binding by these lower-affinity cellular receptors requires that multiple IgGs are presented in immune complexes or on opsonised targets, such as virally infected cells.

The analysis of antibody responses and evaluation of mAb in macaques has provided useful insights into possible roles of Fc/FcR interactions in resistance to HIV as described in the following sections. However it should also be noted that despite much similarity there are potentially important differences in diversity, genetics, and biology of antibody and of FcRs in macaques compared to humans which are mentioned below and in [29-33]. Thus, the extrapolation of FcγR-mediated antibody functions from these models to efficacy in humans should be made with caution. None the less several studies have suggested major roles for the FcRs in effective antibody responses.

2. ANTIBODY FC FUNCTION IN PASSIVE TRANSFER, VACCINE AND NATURAL INFECTION STUDIES

2.1. In Vivo Studies I: Macaque and Mouse Models

A landmark in vivo study in 2007 planted the idea that FcγR-mediated antibody functions will play an important part in HIV therapy. The passive transfer of a bNAb, b12, conferred protection to a SHIV mucosal challenge of macaques, but this protection was substantially reduced when the bNAb was engineered to reduce FcγR-dependent effector function [34]. Thus the protective effect of the bNAb was substantially contributed by the FcγR binding activity of the antibody. Follow up studies using a more physiological exposure of repeated challenge with low virus doses could be resisted with the FcγR-active form of b12 at a 25-fold lower concentration than required for 90% neutralisation [35].

Likewise in macaque vaccination and challenge experiments, IgG effector functions, such as ADCC/ADCP, have been shown to be integral to protection [36] or viremic control [37]. A high-dose SHIV162P3 challenge study showed that immunisation with gp120-CD4 fusion protein (FLSC) elicited titres of CD4i specific Abs [38], in particular co-receptor binding site specific antibodies [39] that correlated with control of viremia. In what is a presumably more physiological repeated low dose challenge of animals vaccinated across varied FLSC/adjuvant regimens, protection from acquisition was correlated with CD4i antibody and ADCC activity, but only in individuals with low CD4 T cell responses. The negative impact of T cell activation, reminiscent of human vaccine studies [40], confounded otherwise protective levels of ADCC antibodies [41].

One study aiming to improve vaccine efficacy used an ALVAC-SIV prime with a boost consisting of a gp120-CD4 fusion protein that displays CD4 binding induced epitopes. While this boosting regimen increased antibody responses to the C1 region and V2 loop, with increased ADCC activity, there was not improved protection against rectal SIV challenge compared to the gp120 boost. In the gp120 boost the V2-peptide antibody response in the mucosa correlated with protection, while for the gp120-CD4 boost serum V2 antibodies correlated with protection [42].

Recently, a human leukocyte reconstructed mouse model was developed to assess antibody mediated protection by neutralisation [43] and activating FcγR-dependent mechanisms [44]. The full potency of bNAbs in protection and control of viremia has been shown in this model to require FcγR-dependent extra-neutralising activity [44]. Cocktails of bNAbs can suppress viral rebound in this infection model in an FcγR-dependent manner. In one study, 44 days after treatment with the cocktail of bNAbs, 1 of 21 “humanised” HIV infected mice exhibited viral rebound while a cocktail of bNAbs engineered to lack FcγR binding was less effective, with viral rebound occurring in 9 of the 15 infected mice. This model has also proved encouraging in a “cure” experiment showing treatment with inducers of viral transcription that re-activated latent viral reservoirs combined therapeutically with the cocktail of bNAbs to suppress viral rebound in about half the infected mice [45].
2.2. In Vivo Studies II. Human Vaccine and Antibody Trials

A second pillar of evidence that FcγR-mediated antibody functions will play an important role in combating HIV comes from the only effective human vaccine trial. The trial, based on ALVAC-HIV Canarypox vector prime containing Env subtype A/E and Gag followed by AIDSVAX HIV-1 envelope protein subtype A/E and B boosting vaccines, was conducted on 16,402 individuals in Thailand and produced a 31.2% protective capacity [46]. While both CTL and antibody responses were induced, both the CTL response and the neutralising antibody responses were relatively weak [47]. Rather than broadly neutralizing antibodies or CD8+ T cells, protection in the trial correlated with a profile of ADCC/Fc-effector functional antibodies in the absence of an IgA response. [46, 48, 49]. A comparison between the prime-boost RV144 vaccine and previous failed vaccine studies, found the RV144 regimen differed by having the capacity to induce higher levels of IgG antibodies capable of mediating FcγR functions, such as ADCC and ADCP [50].

When RV144 and the unsuccessful VAX003 trial were compared, RV144 elicited multi-functional, non-neutralising Abs skewed to higher IgG3 and IgG1 titres, whereas VAX003 elicited monofunctional Abs skewed to IgG4 selection [50]. As IgG3 and IgG1 antibodies have higher affinities for FcγRs, this was consistent with a stronger Fc-mediated response being involved in protection in the RV144 trial. Many of the ADCC active antibodies that recognised gp120 could be competed with the archetypical ADCC mAb A32 [51]. Sieve sequence analysis of HIV isolates from infected RV144 participants tested if vaccine responses selectively blocked infection with particular viral genotypes. Vaccine induced V2-Abs bound conformational epitopes and to a linear epitope of V2 residues 169-182 [52] or 165-178 [53]. The sequence of the V2 loop of the transmitted viruses differed from the vaccine Env such that vaccine efficacy reached 48% against viruses matching the vaccine at lysine-169 [52, 54]. This supported the idea that the transmitted breakthrough viruses in vaccinees were selected by ADCC Abs elicited by the vaccine. Since ADCC Abs are relatively easily elicited compared to bNAbs [55-58] and characterised mAbs such as CH58 and CH59 are not extensively hypermutated, the V2 loop is a possible immunogen for developing FcγR functional antibody responses [56]. Furthermore the V2 loop has been successfully scaffolded in immunisation studies that elicited ADCP active antibodies in rabbits, whereas immunization with gp120 did not raise ADCP functional Abs [59].

Although unsuccessful overall, the vaccine trial VAX004 showed an inverse correlation between ADCVI activity and risk of infection [60]. Furthermore, an IgG4/2 skewed response negatively impacted on protection [61]. IgG4/2 responses are negative indicators of ADCC and ADCP activity and effector activity is increased following the removal of IgG4 from the Ab pool [50]. ADCD mediated by Natural Killer (NK) cells and ADCP by macrophages and neutrophils have since been further described as important mediators of immunity in the RV144 vaccine response [48, 50, 62, 63].

A recent patient treatment study suggests FcγRs may play a role in accelerating the development of anti-HIV bNAbs. In contrast to the slow acquisition of neutralisation capacity in natural infection [11, 64], passive transfer of the bNAb 3BNC117 to viremic individuals off ART had a vaccine-like effect, stimulating Ab responses capable of neutralising heterologous tier 2 viruses. Thus, extra to its neutralising activity, the bNAb 3BNC117 directs the humoral immune response, possibly by engaging Fc receptors on antigen presenting cells [65]. Similar ‘vaccine-like’ effects have been noted with the generation of host anti-tumor immunity following treatment with anti-CD20 Abs. The vaccine effect in this tumor model in FcγR-humanized mice suggested antigen presentation by the therapeutic mAb resulted from its formation of immune complexes with its cognate antigen that bound FcγRIIa on dendritic cells [66]. These studies suggest that Fc-mediated antigen presentation may form a critical feature of optimised HIV vaccines. In addition to its vaccine-like effect, mAb 3BNC117 also cleared infected and ART treated CD4 T cells adoptively transferred to immunodeficient mice in an FcγR-dependent manner [67]. Thus this bNAb has multiple FcγR-dependent anti-viral effects in addition to neutralisation of virus.

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Table 1. IgG subclass binding profiles*.  

<table>
<thead>
<tr>
<th>CD designation</th>
<th>Receptor (allelic form)</th>
<th>Affinity Category</th>
<th>Approximate affinity for IgG3 (M⁻¹)**</th>
<th>Relative binding hierarchy</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD64</td>
<td>FcyRI</td>
<td>High</td>
<td>~10⁻⁵</td>
<td>IgG3 &gt; IgG1 &gt; IgG4</td>
</tr>
<tr>
<td>CD16a</td>
<td>FcyRIIa (V169)</td>
<td>Low</td>
<td>~5x10⁻⁶ M⁻¹</td>
<td>IgG3 &gt; IgG1 &gt;&gt; IgG2</td>
</tr>
<tr>
<td>CD32a</td>
<td>FcyRIIa (E158)</td>
<td>Low</td>
<td>~5x10⁻⁶ M⁻¹</td>
<td>IgG3 &gt; IgG1 &gt;&gt; IgG2</td>
</tr>
<tr>
<td>CD32c</td>
<td>FcyRIIc</td>
<td>Lowest</td>
<td>~10⁻⁴ M⁻¹</td>
<td>IgG3 &gt; IgG1 &gt; IgG4</td>
</tr>
<tr>
<td>CD32b</td>
<td>FcyRIIb</td>
<td>Lowest</td>
<td>~10⁻⁴ M⁻¹</td>
<td>IgG3 &gt; IgG1 &gt; IgG4</td>
</tr>
</tbody>
</table>

*For a detailed review of binding specificities and affinities see (Hogarth & Pietersz, 2012; Wines et al., 2016; Bruhns et al., 2009).

** Note that published affinities can vary but IgG3 is universally the IgG subclass with greatest affinity for any given IgG Fc receptor irrespective of Affinity Category (Powell et al., 1999, Bruhns et al., 2009).
2.3. In Vivo Studies III: Natural Infection

The third pillar of evidence for the protective action of HIV-specific FcγR-activating antibodies has been their association with slowed HIV-1 progression across a range of studies spanning 2 decades [13, 16, 36, 57, 68-70]. Analysis of the rare HIV-1 infected subjects who naturally completely control viral replication (termed “elite controllers”) also illustrate the integral role that ADCC and ADCP play in natural immunity. The trend observed in several studies of natural infection was for higher levels of ADCC antibodies and a response skewed to IgG3/1 subclasses in HIV-1 controllers compared to an IgG4/2 subclass skewing in viremic individuals [18, 61, 71, 72]. ADCC antibodies are produced more rapidly following infection than neutralizing antibodies, which typically take months or years to develop [46, 49]. Similar to what was observed in an analysis of the RV144 trial [50] analysis of elite controller antibody responses suggests these patients are not distinguished by a superior activity in a single Fc functional assay, but rather by activity across a number of different functional assays [61, 73].

Colostral IgG of HIV infected mothers exhibit ADCC activity and are directed against multiple regions of gp120 including CD4i and V1/V2 loop specificities [74]. ADCC functional antibodies have also been associated with protection in maternal-infant passive transfer studies when present in breast milk [75, 76].

In sum, although these diverse studies show that in vivo FcγRs participate in anti-viral mechanisms of protection, the role of individual receptors requires further elucidation. A brief description of the possible roles of individual FcγRs follows.

3. FcγRs AND HIV-1

3.1. FcγRI, the High Affinity FcγR

FcγRI (CD64) expression is induced by IFNγ under pro-inflammatory conditions, such as acute HIV infection [77], on monocytes, macrophages, neutrophils, dendritic cells and mast cells [29]. FcγRI is the high affinity receptor for IgG, binding monomeric IgG and small immune complexes and has a specialised function in the endocytosis [78] and inactivation of toxins [79]. These activities may relate to its activity in ADCVI with anti-HIV polyclonal and monoclonal IgGs [20], wherein the levels of HIV virion opsonisation with IgG may be limiting. Several in vitro studies indicate a particular role for FcγR in patient antibody mediated ADCVI. The inhibitory titre of the anti-gp41 mAbs 2F5 or 4E10, was increased up to 300-fold above neutralisation titre in single round infection studies in monocyte derived macrophages and was dependent on FcγRI [20]. Subsequent investigation of this FcγRI-dependent inhibition with a panel of mAbs found non-neutralising mAbs specific for the V3 loop or gp41, but not other specificities, were able to inhibit macrophage infection [80]. This enhancing property of FcγRI was further confirmed when the expression of FcγRI, but not FcγRIIa or FcγRIIb, in TZM-bl cells conferred greater inhibitory potency on the mAbs 2F5 and 4E10 [81]. FcγR activity, though undefined, was also found to inhibit infection of Langerhans cells and interstitial dendritic cells, early mucosal targets of infection in vivo [82].

FcγRI has a role in antigen presentation at low concentrations of IgG, as antigen presenting cells from FcγRI-deficient mice were impaired in stimulating antigen specific CD4 memory T cell proliferation. However the function of FcγRI in humoral immunity appears to be regulatory as the end effect on B cell mediated immunity in FcγRI deficient mice was increased IgG titres to both soluble and cellular antigen [78].

Unlike FcγRII and FcγRIII, FcγRI is not polymorphic in humans, however at least 3 variants have been identified in the rhesus macaque [33, 83] but the IgG subclass binding specificity and affinity are near equivalent for the rhesus [33] and pig-tailed macaque [29].

3.2. FcγRIIa, a Ubiquitous and Polymorphic Receptor

FcγRIIa (CD32a) is the most widespread and abundant of the activating IgG receptors, being present on all leukocytes and platelets with the exception of T, B and NK cells [26, 84, 85]. FcγRIIa is a key FcR in myeloid cell effector responses [86, 87]. FcγRIIa expressed on macrophages mediates ADCP of opsonised infected cells and further stimulates the secretion of anti-viral factors such as TNFα and IL-1beta [88].

FcγRIIa genetic polymorphisms are highly relevant to human disease. The High Responder (HR) and low responder (LR) polymorphism at amino acid position 131 [89] is a highly significant risk factor for SLE [90-92], idiopathic thrombocytopenia [93] colitis [94] and rheumatoid arthritis [87, 95, 96], malarial parasitemia levels [97] and aggressive periodontal disease [98]. The protective effect of the H131 polymorphism for severe infection by encapsulated bacteria relates to this receptor being the only human FcR that functionally binds IgG2 [99].

The influence of FcγRIIa polymorphisms on HIV acquisition or disease progression may be obscured by variations between patient cohorts, differing IgG subclass responses, particularly IgG2, as well as small patient numbers in some studies. This receptor plays a broad role in immunity, encompassing antigen presenting cell function (e.g. [66]) and Th17 cell development (reviewed in [7]). Two studies have shown an association between the presence of at least one H131 allele and improved outcomes. In one study, homozygosity or heterozygosity for H131 was associated with slower progression to a CD4+ cell count <200 cells/mm³ [100]. The second study, conducted by French et al. (2010) [19], showed an association between these genotypes and lower HIV replication during interruption of ART following vaccine administration [19]. In this study, patients with IgG2 anti-p24 responses also exhibited lower HIV replication. Although the H131 form of the receptor is associated with marginally increased affinity for IgG1 and IgG3, it is more likely its necessity for IgG2 binding that contributed to improved outcomes in this trial.

However, the former of these two studies also observed that individuals with a homozygous H131 genotype were at greater risk of developing pneumonia as an AIDS-related infection. Conversely, other studies have found no associa-
tion between this variant and vaccine-induced protection when analysing the RV144 and VAX004 trials [101].

Monocyte FcγRIIa-dependent phagocytosis has been implicated in the superior activity of antibodies from HIV controllers and untreated chronic progressors in an assay that comprised mixed autologous effectors (monocytes, NK cells) and HIV infected CD4 targets and in an assay of gp120-coated bead phagocytosis by the THP1 cell line [18]. Antibodies from HIV infected individuals have increased phagocytic activity over time of infection [102]. A further study using the THP1 monocyte cell line and gp140 coated beads and a methodology that quenched fluorescence of surface bound but not internalised beads, found the RV144 vaccinees lacked antibodies active in phagocytosis.

FcγRIIa may contribute to protection against acquisition as FcγRIIa on immature dendritic cells is responsible for the enhanced inhibition by anti-Env antibodies of infection of these cells [80]. Furthermore, the optimisation of bNAb VRC01 for binding to FcγRIIa improved phagocytosis by macrophages and neutrophils, two key innate effector types at mucosal sites [103].

FcγRIIa is unique to primates but evaluating antibody function in the macaque is nonetheless not straightforward as ligand binding interactions of this receptor differs in the human and the macaque. The pig-tail [32] and rhesus macaque receptor [33] has lower affinity than the human counterpart and a low activity form (Pro-131) occurs in both macaque species. At least eight variants of FcγRIIa have been observed in a small cohort of pig-tailed macaque [32] and five in rhesus macaques [33, 83].

3.3. FcγRIIb is a Key Regulator of Ab-Mediated Immunity

FcγRIIb (CD32b) has highly homologous IgG binding ectodomains to those of FcγRIIa but is an inhibitory receptor. FcγRIIb regulates cell activation via an ITIM (immunoreceptor tyrosine-based inhibitory motif) within its cytoplasmic domain [104, 105] which recruits SHIP and SHP phosphatases (reviewed in [106]). The FcγRIIb1 variant controls the activation of B lymphocytes by the BCR via co-engagement of the receptor and BCR by antigen-IgG immune complexes. The splice variant, FcγRIIb2, differs from FcγRIIb1 by a shorter cytoplasmic domain and is expressed on myeloid cells, although constitutive expression on cells is limited (e.g. basophils, [86]) such that control of activating FcγR function on myeloid cells by FcγRIIb2, although potentially potent, is probably cell type and cell state specific [107]. Nonetheless, the FcγRIIa-dependent phagocytic activity of antibodies of HIV controllers has been reported to be less regulated by FcγRIIb, and was linked to greater potency of FcγRIIa-mediated phagocytosis by monocytes and THP1 cells [18]. The molecular basis for HIV controller IgG preferentially binding FcγRIIa over FcγRIIb is not elucidated.

The ratio of Ab binding by activating ITAM receptors to inhibitory ITIM receptors is an indicator of the net outcome of their activation or regulation of effector cells [108]. Despite the high homology of the of FcγRIIa and FcγRIIb ectodomains, human FcγRIIb has lower affinity than its activatory counterpart. However, this is not the case in macaques where the binding affinities of IgG to pig-tailed macaque mnFcγRIIa and mnFcγRIIb are reversed when compared with the human receptors [32]. This higher affinity of macaque FcγRIIb and lower affinity of the macaque FcγRIIa raises the possibility of somewhat different regulation of FcγRIIa by FcγRIIb in macaque compared to human myeloid cells.

3.4. FcγRIIc

FcγRIIc (CD32c) is an activating receptor formed by cross over between the FcγRIIb (ectodomain exons) and FcγRIIA (cytoplasmic domain exons) genes (Fig. 1). Only about 20% of Caucasians express a functional FcγRIIc on neutrophils, macrophages and NK cells, and its contribution to antibody function has been little studied [109]. This receptor is not present in the macaque.

An investigation of correlates of protection in the RV144 trial found FCGR2C genetic polymorphism correlated with vaccine efficacy (up to 91%) [110]. A recent report by Peng et al. further investigated these correlates finding that these polymorphisms affected expression of FcγRIIC, FcγRIIa and also Fc like receptor A (FCRLA) [22]. These results suggest a possible functional association between these polymorphisms and warrants future investigation.

3.5. FcγRIIIa, a Key Activating Receptor

FcγRIIIa (CD16a) is expressed on NK cells and phagocytic macrophages. FcγRIIIa is the most abundant FcγR on NK cells which are major mediators of ADCC [16] and signals via the signal transduction subunits FcRγ and/or CD3-ζ [13]. Recent studies of HCMV infection have identified the expansion of adaptive NK cells with altered expression of Syk [111] and down regulation of FcγRIIIa, but which are more potent ADCC effectors and producers of IFNγ [112]. Mechanistically this increased activity has been linked to a switch from FcγRIIIa association with FcRγ/CD3-ζ to CD3-ζ alone by loss of FcRγ expression and synergistic signalling from CD2 which acts as a co-receptor for FcγRIIIa (reviewed in [113]). This population of “ADCC-specialised” NK cells has also been identified in viremic and cART-treated HIV infected patients where it is expanded to comprise 90% of the mature NK compartment [112].

In humans there are two functionally important variants of FcγRIIIa that have either a valine or phenylalanine at residue 158. These polymorphisms affect the affinity of binding of IgG1 and IgG3. Therapeutic responses to anti-tumour IgG1 mAbs correlated with patient expression of the higher affinity (Val158) allele of FcγRIIIa, suggesting the importance of the Fc-FcγRIIIa interaction in the mechanism of action of these antibodies [114, 115]. Consequently, it is well established that this polymorphism enhances Ab affinity and can be associated with increased ADCC function of these therapeutic mAbs. However, understanding the role of FcγRIIIa in HIV control and protection has proven more difficult (reviewed in [9]). In macaques at least three variants of FcγRIIIa have been observed [83].

Some studies of FcγRIIIa polymorphism in HIV pathogenesis, vaccine response, maternal transfer and disease pro-
gression have yielded associations but overall results have been conflicting. One study looking at FcγRIIIa alleles in HIV+ patients found that homozygosity for Val158 was associated with a higher rate of disease progression [116]. On the other hand, a large cohort study observed no association between FcγRIII variants and HIV-1 disease progression [100]. A recent study of HIV-1 in Kenyan women also failed to find any association between polymorphisms of either FcγRIIa or FcγRIIIa and numerous measurements of disease progression [117].

Individuals with low sexual risk-behaviours and V158 homozygosity were correlated with increased infection risk in the VAX004 Trial [101]. Independent analysis of this trial, however, found no association between each FcγRII/IIIa variant alone and risk of infection [118]. However, the combination of homozygosity for the lower affinity forms of both FcγRIIa-FF158 and FcγRIIa-RR131 was associated with higher HIV disease progression in a study by Pandey et al. (2013) [118]. In a study of passive Ab transfer from mother to infant via breast milk, the maternal FcγRIIa-Val158 allele associated with reduced HIV-1 transmission [119]. It should be noted that the infection context here is different than in post-vaccination sexually transmitted infection.

The conflicting results of studies of FcγRIIIa variants suggest caution in the interpretation of its role in HIV vaccine responses, infection and disease progression. One possible explanation is that although the higher affinity Val158 form may induce heightened NK ADCC efficacy, this could also contribute to increase total immune activation which could be deleterious in HIV acquisition.

3.6. The Influence of IgG-Fc Glycosylation on FcγR-Mediated Function

Tumour immunotherapy has driven the development of therapeutic Abs with their Fc glycosylation optimised for interaction with FcγRIIIa and ADCC by NK cells. Analysis of the glycosylation of the Fc found that the absence of a bisecting fucose in the Asn297-linked carbohydrate greatly increased interaction of the Fc with FcγRIIIa [120-123]. Thus, therapeutic Abs that lack this fucose have been shown to have improved receptor binding and target killing functions [124, 125], although mechanisms for improved antitumour outcomes in the clinic are multifactorial [126].

The therapeutic efficacy of a glycans-engineered form of the archetypal anti-HIV bNAb b12 has proved unclear. The nonfucosylated variant of mAb b12 (Nf1b), increased FcγRIIa binding and 10-fold improved potency in NK-mediated ADCC and PBMC-mediated ADCVI assays, but, contrary to expectation, proved in vivo no more potent than wild-type Ab in protecting SHIV challenged rhesus macaques [127].

It is noteworthy that the enhancement of anti-HIV mAb b12-Fc binding to FcγRIIa has been reported to lower function in assays presumably dependent on different receptors [73]. A similar outcome has been reported previously for the switch away from neutrophil/FcγRIIa-mediated ADCC to exclusive NK/FcγRIIa-mediated ADCC for the FcγRIIa-binding enhanced anti-tumor mAb Cetuximab. FcγRIIa engagement plays a prominent role in neutrophil activation and the FcγRIIa-binding enhanced mAb engaged neutrophil FcγRIIib in preference to FcγRIIa [128]. As already noted neutrophils may be important innate effectors at mucosal sites [103].

Interestingly, IgG antibodies with short glycans, agalactosylated “G0” forms and fucosylated forms in the Fc have recently been found to interact with Mucin-16. This large protein extends 200 nm above the mucosal epithelial surface and complexes with IgG-Fc. Mucin-16, and possibly other mucins, are thus proposed to improve Ab specific trapping of antigens in the mucus barrier and extracellular cleavage of crosslinked mucin-16 is likely to shed trapped virus [129]. Some of these glycoforms of Fc interact poorly with FcγRIIa (e.g. fucosylated [120-123]) and so are possibly non-inflammatory versions of IgG that may be most appropriate for protection against mucosal challenge, especially considering the lack of evidence for improved protection in SHIV challenge studies by the non-fucosyl-variant of the b12 bNAb [127].

4. HIV EVASION AND SUBVERSION OF FcγR-MEDIATED Ab FUNCTIONS

HIV efficiently evades the host protective antibody response. Envelope sequence diversity, glycosylation and conformational masking of conserved functional sites, together with low levels of Env protein expression on the virion and the infected cell surface all contribute to this evasion [130]. HIV nef and vpu also downregulate CD4 surface expression on infected cells thereby preventing exposure to ADCC antibodies targeting CD4-induced (CD4i) epitopes on Env [131]. HIV not only evades but, recent in vitro studies indicate, HIV also subverts host humoral immunity. This occurs by shed gp120 binding to CD4 on uninfected CD4 T cells which then become opsonised by CD4i specific anti-gp120 Abs. As such uninfected cells express high CD4 levels, they are efficiently killed by ADCC [132]. Since almost half of anti-HIV antibody specificities may be directed against CD4i (e.g. A32-like Abs, [39, 133]) this bystander killing by ADCC may comprise a significant component of the loss of CD4 T cells and disease progression.

Indeed a recent ADCC study found the A32 epitope to be poorly expressed on target cells infected with a primary HIV isolate but well expressed and effective for ADCC on targets infected in vitro with a lab adapted HIV strain. It was suggested this difference arises from the unligated Env trimer of the primary strain having stringent conformational integrity that restricted Ab reactivity to bNAbs, while the lab adapted Env had a ‘looser’ conformation that exposed a greater range of epitopes including CD4i epitopes [134]. If this proves to be generally true with primary isolates it argues that the CD4i epitope-specific Abs may not contribute to host defence but rather are co-opted by the virus and participate in the decline of CD4 T cells.

This picture is at odds with challenge studies in macaques vaccinated with a gp120-CD4 fusion protein, that efficiently displays the CD4i conformation of gp120. In these studies the CD4i specific Abs elicited are clearly protective (e.g. [41, 42]). Furthermore, a recent microscopy study showed fluorescently labelled virions bound to the
target cell surface displayed Env epitopes, including CD4i and CD4 binding site epitopes, although this reactivity was lost as entry progressed. It is notable that the level of opsonisation may be modest as 3-6 A32 mAb molecules were estimated to be bound to each cell bound virion [135].

The conformation of the CD4i epitope has been captured in a truncated and stabilised gp120 innerdomain mini-protein which binds A32 and other CD4i specific mAbs [136]. This has been suggested to be a potential vaccine candidate for eliciting CD4i focused antibody responses but will first require an evaluation of the importance of off target killing of uninfected bystander cells to determine if this approach will be effective. Here the multiple effects of small CD4 mimic compounds, themselves highly potent inhibitors of infection [137], may prove useful for redeeming CD4i ADCC by redirecting it to killing infected targets. First, these act with co-receptor binding site Abs to reveal CD4i epitopes on the unliganded Env to make infected cells ADCC targets [138]. Secondly, they have the benefit of inhibiting shed gp120 binding to uninfected CD4 T cells and so sparing them from possible bystander ADCC killing [132]. Third, they sensitise HIV for neutralisation such that otherwise ineffective Ab responses elicited by infection or various immunization regimens, if of sufficient anti-gp120 titre, can effectively neutralise CD4 mimic treated virus [139]. Recently a small compound has been reported that inhibits Vpu mediated degradation of tetherin, providing a novel approach to sensitising infected cells for ADCC [140]. Together such approaches may play a role in eure strategies involving reactivation of latently infected cells by enhancing Env surface expression and revealing CD4i epitopes in Env, thereby sensitising them to elimination by ADCC [21].

5. ASSAYS FOR Fc FUNCTION OF ANTI-HIV ANTIBODIES

The mere presence of detectable opsonizing Ab (i.e. without measure of mechanism) may be a poor correlate with protection compared to mechanistic measures of antibody function. Thus ADCC assays are fundamental components of correlates of protection in the evaluation of current and future HIV vaccine efficacy trials. Given the heightened interest in ADCC in vaccine studies and Fc-mediated functions more generally, there is an urgent need for standardized, scalable, rapid and sensitive assays of immunity to HIV. Unfortunately the routinely employed research-based cell culture-based assays to dissect immune correlates of the RV144 trial and of other large HIV research cohorts, although quantitative, are difficult to standardize.

5.1. What to Measure? Some Considerations in Evaluation of Fc Receptor Function

HIV study outcomes are affected by differing patient populations, infecting viral strains, types of exposure and whether effects in transmission, acquisition or progression are under examination. All these factors affect decisions of how to best evaluate the role of anti-HIV Abs.

As already discussed different IgG subclasses, glycovariants of the Fc, different FcγRs, polymorphisms and interspecies differences all influence the functionality of this ligand receptor system. However, as will be explained below, even fully defining the receptor and antibody alleles, subclasses and glycoforms does not sufficiently account for all the factors that modify the Fc receptor function of IgG antibodies. Since only multivalent IgG immune complexes effectively bind to the low affinity Fc receptors, FcγRII and FcγRIII, the composition and nature of the complexes formed by IgG opsonisation of antigen, or virion or virus infected cell can profoundly influence receptor activities.

The orientation of Fab binding to an epitope can affect FcγR activity. Human antibodies are highly diverse with ~10^{13} possible antigen interactions [141] which, in a normal polyvalent immune response, will generate multiple possible ways of binding an epitope. For example the C1 specific mAbs, N5-i5 and 2.2c, bind to highly related epitopes with similar affinities and yet have a 75-fold different efficacy in ADCC. Partly this difference results from mAb N5-i5 recognising more cell-C4D bound Env but, in addition, 2.2c orients its Fc toward the cell surface, while mAb N5-i5 presents its Fc away from the target cell. Presumably the latter orientation better engages FcγRIIIa and ADCC function [142].

While a number of studies have examined synergistic effects in antibody binding and virus capture, the density of IgG opsonisation is a further factor that may influence the efficacy of FcγR engagement. Because of the multivalent nature of the interaction of FcRs, combinations of antibodies are likely to be superior over individual mAbs alone for the functional opsonisation of targets with low antigen density. IgG opsonisation is limited by the reduced antigen availability resulting from the combined effects of low Env expression by primary viruses, the gp120 glycan shield, conformational masking, sequence variation and the downregulation of CD4 and tetherin. Under such circumstances, the simultaneous occupancy of epitopes on Env may markedly affect FcγR interactions and the quality of subsequent effector cell responses. Indeed some V2 and C1 region specific mAbs elicited in the RV144 trial when paired together showed superior ADCC activity over that of the individual antibodies [143].

The conformational plasticity of Env and the exposure of different epitopes at different points in the virus infectious cycle, as exemplified in the extreme by the differences between bound gp120 and closed Env trimer, result in different forms of Env being optimal for presenting particular epitopes for Ab opsonisation [144-146]. Hence for assays of particular effector functions, whether the Env is native or engineered, in soluble form as monomer or trimer or in the context of a virion or target cell can all affect the outcome of in vitro assays and their potential for correlation with protection in vivo. Further complexity in understanding Ab/FcγR involvement in anti-HIV immunity arises from the engagement of different FcγRs and the varied action of different immune effector cells at different anatomical sites in transmission, acquisition and disease progression.

Presently it is not clear what Fc receptor-dependent function or assay provides the best in vitro correlate to protection or viremic control. Recent studies indicate protection is best indicated by responses that are able to trigger multiple func-
tions [61]. A comprehensive study of seven FcγR-dependent functional assays found that different assays, even when assessing ADCC function, were non-redundant and reflected unique aspects of Ab function. The analysis of multiple ADCC assays in combination best discriminated different patient and vaccinee groups [73].

5.2. Cell Based Functional Assays for FcR Activating Antibodies

Most current cell-based assays of FcγR function of antibodies focus on NK cells, although as discussed other innate leukocytes can be potent effectors, and typically measure the expression of activation markers, the release of cytokines and lytic proteins or the killing capacity of NK cells and use flow-cytometry or ELISpot techniques [147-149]. Such assays include the rapid-fluorimetric ADCC assay (RFADCC) [150, 151], NK viral inhibition assays [68, 152], lactate dehydrogenase release assay [153], granzyme delivery assays [154, 155], and NK cell activation assays measuring interferon-gamma and/or CD107a [13]. Such assays can be long, complex and difficult to standardize or reproduce precisely. Most assays involving donor cells need large amounts of human donor blood as a source of effector cells. There are substantial Fc-receptor polymorphisms across human donors that influence the readouts of these assays adding to issues around variability, standardization and reproducibility across laboratories.

Many assays measure HIV-infected, or gp120-coated, target CD4 T cell killing. Target cells may also be sensitised using inactivated virus [38]. Standard natural cytotoxicity assays measure the release of chromium, ⁵¹Cr, from isotope-labelled unsporised target cells in the presence of NK cells. ADCC is measured by the additional killing of targets in the presence of target specific Abs. This assay was used by Baum et al. (1996) to show that high titres of Abs that mediate ADCC correlated with a slower AIDS progression [69]. Cytotoxicity assays measuring release of europium label from lysed target cells using time resolved fluorescence are replacing the radioisotope label assays [156].

The lactate dehydrogenase (LDH) release cytotoxicity assay, marketed as the CytoTox96 nonradioactive cytotoxicity assay kit (Promega), is a colorimetric alternative to chromium release assays. The assay measures LDH released upon cell lysis and has been used, for example, in recent studies examining the potential of HIV-1 specific ADCC in killing latently infected cells upon viral reactivation [21] [157].

Granzyme B (GrB) is a serine protease that, on delivery by NK cells into the cytoplasm of target cells, initiates apoptotic signalling and cell death in the target. Upon delivery, the enzyme recognises and cleaves a short peptide sequence in procaspases, activating them and so triggering the apoptotic pathway. The GrB assay, marketed by OncoImmunin as GranToxiLux®, measures delivery of the enzyme to target cells, which are preloaded with a GrB targeted substrate that becomes fluorescent upon cleavage. This method was evaluated by Pollara et al. (2011) for measuring the ADCC-mediating capacity of Abs against HIV and SIV in humans and non-human primate models [154].

Caspase-3 is an enzyme activated downstream of GrB and is a key player in the apoptotic process. A second Oncolimmunin assay, PanToxiLux™ measures both GrB and caspase activity. A comparison of the GranToxiLux® and PanToxiLux™ assays by Konstantinus et al. (2016) found that both were both suitable for measuring NK killing and HIV-specific ADCC activity, although the GranToxiLux® assay had improved detection of HIV-Specific NK cell mediated ADCC activity [155]. A T lymphoblastoid cell line, CEM.NK® CCR5, that is resistant to natural cytotoxicity of NK cells and expresses both CCR5 and endogenous CXCR4 [158] has been the basis of many assays for HIV replication, neutralisation and ADCC.

The Rapid Fluorometric ADCC (RFADCC) assay developed by Gomez-Roman et al. (2005) involves the dual labeling of target cells (e.g. CEM.NK® CCR5) with the cell membrane marker PKH-26 and a cytoplasmic marker (e.g. CFSE) with the former stain marking targets and the loss of the later stain indicating target killing. This assay has been used to demonstrate that ADCC activity of anti-gp120 Abs elicited in macaques by vaccination correlated with reduced viremia following in vivo SHIV mucosal challenge [37, 150]. Further evaluation of this assay found that monocytes were in fact the main effector cell in PBMCs and trogocytosis occurred with target cell membrane being incorporated into the monocytes [159]. The RFADCC assay has also been used to evaluate RV144 vaccine induced ADCC antibodies showing immune selection of viruses by these Abs [160]. In a modification of RFADCC approach EGFP was expressed in the CEM-NK® cell line with dual staining by propidium iodide marking killed target cells [161].

Another format of ADCC assay uses CEM.NKR cells expressing high levels of CCR5 that were transduced with a Tat stimulated HIV promoter driving luciferase expression. Killing of the infected CCR5-sLTR-Luc cells is measured by loss of luciferase activity which is specific for infected targets [162]. In another assay, target cells are labelled with a viable cell stain (Far Red DDAO cell tracker, Life technologies), infected with virus, ADCC performed and killed infected targets enumerated by flow cytometry as intracellular stained gag-positive and cell tracker dye-negative. The same study used an infected target specific assay where CEM-NKR cells were infected with recombinant virus incorporating IRES-GFP. ADCC was performed in the presence of the fluorescent nuclear stain DAPI, Abs and effector cells with the lysed targets GFP+ and becoming DAPI+ as visualised by live imaging. This demonstrated bNAbS did not cause uninfected bystander killing [11].

Rather than measuring target killing, NK activation assays can be used as a surrogate for ADCC activity. They involve the quantification by flow cytometry of cytokines or markers produced as a result of NK activation. Chung et al. (2009) used this technique to measure the expression of intracellular IFNγ and surface CD107a on NK lymphocytes but found poor correlation between IFNγ-expression and killing in the RFADCC assay [13].

A commercial (Promega) assay for activation of FcyRIIa as a surrogate for ADCC uses a Jurkat T cell line expressing a NFAT binding promoter controlling luciferase expression. This assay was used to demonstrate Vpu regulation of
tetherin expression inhibits ADCC of infected target cells [163] and that, in macaques persistently infected with an attenuated SIV, acquisition of protective immunity correlated with ADCC activity [55].

Following infection of target cells at low m.o.i., ADCVI assays measure the decrease in viral replication (e.g. levels of p24) in the presence of anti-HIV antibodies and effector cells. Forthal et al. (2001) found plasma from acutely infected patients and normal NK effector cells were able to inhibit viral replication, suggesting a role for antibody and NK cells in early control of infection [68]. The assay can use NK cells or monocytes for killing of infected targets [18]. Other formats measured Fc-dependent direct inhibition of replication in primary macrophages [20].

Ab-dependent phagocytosis assays can use either opsonised virions, beads or target cells. Phagocytosis of large targets requires actin reorganisation. Although described as phagocytosis Ab dependent uptake of opsonised virus is more accurately described as endocytosis. For example, the phagocytosis (or endocytosis) of FITC labelled SIV was found to be equivalent when the SIV was opsonised by IgG elicited by either gp120 or CD4-gp120 boost vaccination regimes [42]. Conventional and imaging flow cytometry has shown a hierarchy of IgG3 > IgG1 > IgA for the internalisation of fluorescent virus by monocytes [164]. Assays can use distinct labels to distinguish internalised from surface bound opsonised virus or beads. In one such study HIV infection was found to elicit a humoral response able to mediate THP-1 cell phagocytosis of gp140 coated beads, in contrast with the RV144 vaccine response which was inactive [102].

In the absence of knowing the most appropriate effector function on which to focus, a general measure of FcγR activating capacity has clear utility.

5.3. FcγR Binding Assays

BLAcore assays (e.g. [27, 28]) have been used to survey the binding activities of the different FcγRs with IgG subclasses in 1:1 stoichiometric interactions. The normal ligands for cellular FcγR interactions are complexes of IgG and increased complex size increases the avidity of interaction and binding activity [165]. Therefore more functional antigen-specific assays measure binding of complexes to immobilised recombinant soluble receptors [18, 166]. For example, recombinant soluble FcγR ectodomains with c-terminal hexahistidine tags have been immobilised on plates via anti-hexahistidine mAb [166] or nickel [18] to produce an array of receptors for binding IgG ligands. A powerful high throughput-put assay used receptor coupled beads with antibody binding detected using PE-conjugated anti-human IgG [167]. While these assays give valuable information about immune complex:FcγR binding, the stoichiometries of the interactions are not well defined. Another high through-put bead format is an ‘alpha screen’ competitive binding assay (Perkin Elmer) which has been used to evaluate the affinities of mutant IgGs for FcγR.

5.4. Dimeric rsFcγR Assays

We developed a simple, rapid and scalable biochemical approach to evaluating FcγR-dependent antibody efficacy in innate effector activation. The assay exploits the intrinsic low affinity interactions of FcγRII and FcγRIII by using the avid binding of recombinant ectodomain homodimers Fig. (2). The low affinity Fc receptors, FcγRII and FcγRIII, have $10^{-6}$ to $10^{-7}$ M$^{-1}$ affinities for uncomplexed monomeric IgG1 and functionally bind IgG opsonised targets and antigens by virtue of the avidity of multiple Fc regions of IgG opsonised targets binding receptors arrayed on the effector cell surface. Clustering of these receptors leads to their phosphorylation and thereby innate cell activation, and effector function.

The rationale of our approach to evaluating antibody responses was to exploit the fundamental multivalent aspect of Fc receptor function whereby avid interactions underlies binding, cell activation and ultimately effector function. As the minimal unit of an avid interaction is a dimer we constructed homodimers of the ectodomains of the low affinity FcγRs, FcγRII and FcγRIII that were linked by their respective membrane proximal stalks as single polypeptides. Since the stalk regions of the cellular receptors allow some freedom of movement of the ectodomains we reasoned this to be a suitable constraint by which to tether the ligand binding portions of the dimer. As the monomeric ectodomain of the receptors do not bind stably, the observed binding of the dimeric rsFcγR ectodomains can be simply interpreted as two IgG antibodies being presented with their Fc regions sufficiently close to be bridged by both ligand binding modules of the engineered dimeric rsFcγR [168]. Thus, while affected by the quantity of bound IgG, as might be measured with a polyclonal anti-IgG, the binding of dimeric rsFcγR to IgG opsonised antigens indicates the favourable proximity/orientation of Fcs. Examination of pooled antibody responses to H1N1 hemagglutinin with dimeric rsFcγRIIIa found increased reactivity with IgGs produced subsequent to the H1N1 pandemic, suggesting a greater occupancy of strain specific epitopes upon HA opsonisation with these IgGs. Furthermore, anti-HA antibody activity in the dimeric rsFcγRIIIa assay correlated with NK activation. Studies with HIV infected patients using the assay have found indication of superior dimeric FcγR binding activity in elite controller
sera and conversely diminished activity in Env specific antibodies from cART patients (Vijaya Madhavi, unpublished results). This high-throughput dimeric rsFcR binding ELISA format could find substantial utility in the rapid evaluation of large numbers of HIV vaccine trial samples.

CONCLUSION

Ample in vivo data show Fc receptor-mediated functions of anti-HIV IgG play an important, though partially effective, role in natural and vaccine-induced immunity. Optimised Fc functionality will comprise some part of the mix that achieves an HIV cure. The predictive value of in vitro tests for correlating with in vivo protection will improve with future matching of appropriate Fc receptors, cell types, antibody forms and functional readouts to in vivo protective functions. While NK cell/FcγRIIIa-mediated ADCC will remain a staple for evaluation of antibody immunity, particularly for control of viremia, other assays, e.g. focused on antibody function in the mucosa, may be more predictive of protection against acquisition. The assay described herein is, to our knowledge, the first attempt to develop a simple in vitro FcγR assay that, in addition to retaining all the FcγR intrinsic properties, such as IgG subclass and glycoform binding selectivity, is sensitive to the nature of the complex formed between antigen and antibody. Key features of this are the density of IgG occupation of adjacent epitopes and the orientation of the Fc regions. The ease of this dimeric FcγR ELISA assay lends itself as a useful, high-throughput screening method for Fc-mediating antibodies in future HIV vaccine trials and studies of HIV-1 immunity.

LIST OF ABBREVIATIONS

ADCC = Antibody dependent cellular cytotoxicity
ADCP = Antibody dependent cellular phagocytosis
ADCVI = Antibody dependent cellular virus inhibition, bNAb, broadly neutralizing antibody
FcR = Fc receptor
rsFcR = Recombinant soluble FcR

CONFLICT OF INTEREST

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