

HIV-Specific Antibody Immunity Mediated Through NK Cells and Monocytes

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Abstract: The partial success of the RV144 trial re-energized the field of HIV vaccine research, which had stalled after vaccines based on neutralizing antibody and cytotoxic T cells had failed to induce protection. A large post-vaccine research effort has focused attention on the role of non-neutralizing antibodies in the protection afforded by the RV144 vaccine. These binding antibodies can initiate immune responses such as antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) and combine elements of the adaptive and innate immune system in the form of antibodies and effector cells (including NK cells, monocytes and granulocytes). A complex interplay exists between the variable portion of the binding antibody and its HIV antigen target on one hand and the constant region of the antibody and the Fc γ -receptor of the effector cell on the other hand. Technical advances have revolutionized the abilities of scientist to detect the targets of non-neutralizing antibodies, including both envelope and non-envelope epitopes, and their role in forcing escape. Our understanding of the antibody characteristics (including IgG subclasses and Fc glycan profile) is providing valuable insights into their optimal structure and function. We expand on critical research on ADCC effector cells, particularly education of NK cells. We introduce the concept of HIV antibody-dependent trogocytosis by monocytes as a potentially important aspect of HIV immunity. In summary, this review highlights recent advances in HIV-specific antibody immunity mediated through NK cells and monocytes.

Keywords: ADCC, antibodies, HIV, KIR, monocytes, NK cells, vaccines.

1. HIV NEUTRALIZING ANTIBODIES

1.1. HIV Neutralizing Antibodies and their Protective Role

The development of an antibody (Ab) response against HIV is mainly directed against the envelope (Env) (gp120 and gp41), the core (p24) and the matrix (p17) proteins and anti-HIV Abs become detectable within a few weeks after infection [1]. Neutralizing Abs prevent virus infection by binding with their variable region to the viral surface and blocking of entry into the host cell (Fig. 1A).

Only about 25% of HIV-infected individuals develop broad neutralizing Abs (bnAbs) (reviewed in [2]) that can inhibit infection by the vast majority of HIV-1 strains. Further, bnAbs usually require years to develop due to the requirement for Ab maturation. The bnAbs described to date exhibit high levels of somatic hypermutations of up to 30% and some but not all bnAbs have long third complementary-determining region (CDR-H3) of ≥ 20 aa [3-5]. Even in subjects that develop bnAbs, these Abs are usually not effective against the current circulating virus [6]. bnAbs isolated from HIV-infected individuals [7-11] mainly target the following epitopes: the conserved CD4 binding site (b12, VRC01-3 [12-15]), mannose cluster on gp120 (2G12, PGTs [9,16]), gp41 MPER region (2F5, 4E10, 10E8 [17-19]) and the V1/V2 and V3 region (PG9/16, PGTs [9,20]) (reviewed in [21,22]). Further characterization of the bnAbs 2F5, b12

and 2G12 has shown that they also have potent ADCC activity *in vitro* [23-26].

The protective potential of bnAbs has been shown in passive transfer studies in rhesus macaques, in which bnAbs provide sterilizing protection against the simian-human immunodeficiency virus (SHIV) [27-33]. Passively administered bnAbs have also been shown to delay HIV-1 rebound after cessation of cART [34]. Unfortunately, HIV bnAbs are difficult to elicit by standard vaccination regimes as they are unable to recapitulate the Ab maturation features required for generating bnAbs [5]. Despite intensive efforts over the last 3 decades, all human vaccines tested before 2009 failed to introduce bnAbs. No nAbs using AdV5 vaccine modality [35] and only very narrow nAbs using gp120 protein vaccines [36-38] were induced in human vaccine efficacy trials to date (reviewed in [39]).

2. OTHER ANTIBODY FC-MEDIATED IMMUNE RESPONSES - FOCUS ON ADCC

2.1. Non-Neutralizing Abs

Neutralization is often considered as the most efficient mode of Ab-mediated defense against viral infections [29]. However, Hessel *et al.* demonstrated that HIV bnAbs, mutated in their Fc region to abrogate their ability to mediate Fc-mediated immune functions, exhibited reduced ability to prevent infection of macaques with SHIV [40,41]. This highlights that not only neutralization but also Fc-mediated immune functions are important for protection from HIV. Further, virus-specific non-neutralizing binding Abs may also have a substantial impact on preventing HIV infection and/or clearing virus particles or virus-infected cells [42]. In

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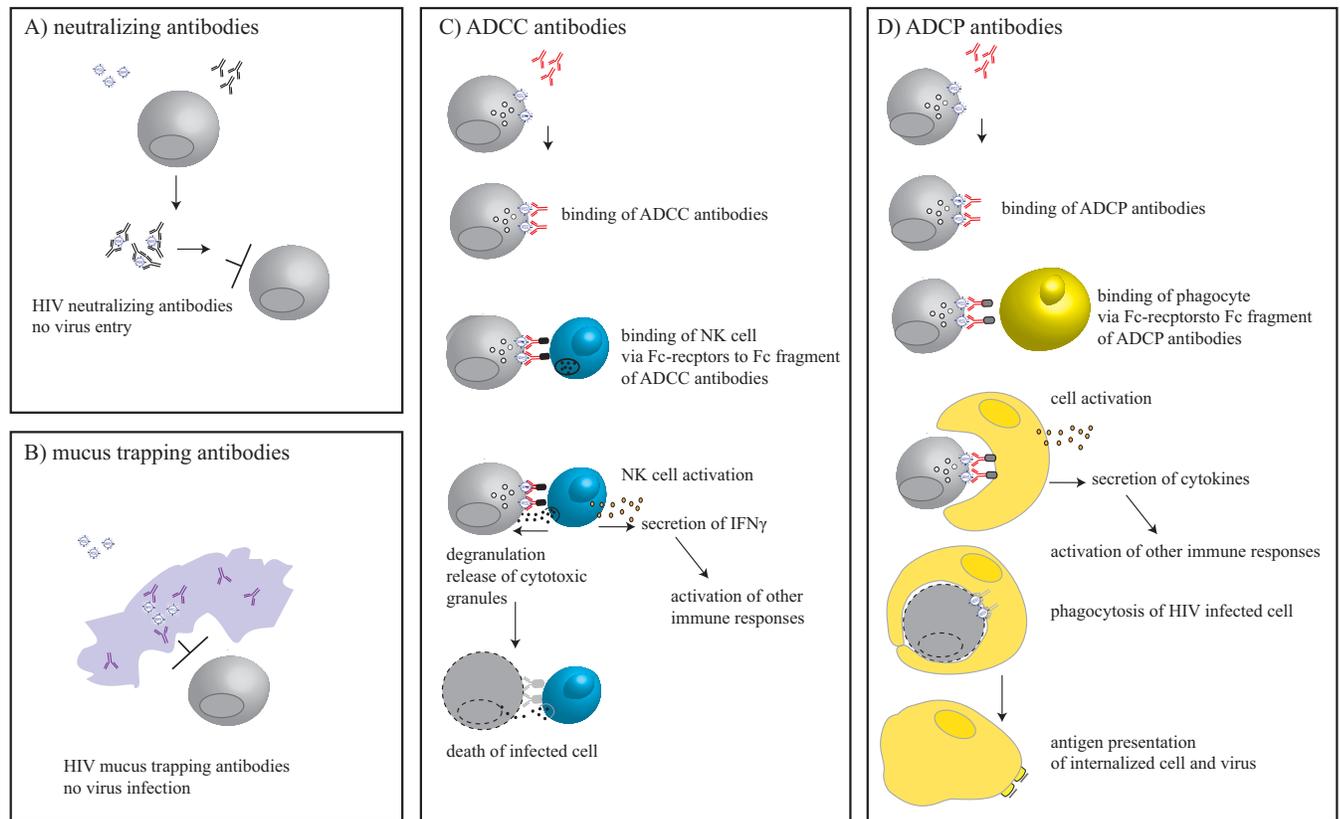


Fig. (1). The different modes of action of anti-HIV antibodies. (A) HIV neutralization and inhibition of attachment and/or fusion of the virus with the cell membrane. (B) immobilization and trapping of HIV in mucus by binding of antibodies, (C) antibody-dependent cellular cytotoxicity (ADCC) resulting in NK cell (blue) degranulation and cytolysis of HIV-infected cells (grey) as well as secretion of cytokines that can exert paracrine or autocrine effects and (D) antibody-dependent cellular phagocytosis (ADCP) of the HIV-infected (grey) cell by phagocytes (yellow) resulting in elimination of infected cell, virus antigen presentation and cytokine secretion by phagocyte.

contrast to the development of rare bnAbs, essentially all HIV-infected individuals develop non-neutralizing Abs during the acute phase of HIV, generally before the induction of bnAbs. Abs can stimulate innate immune cells to mediate effector functions through the binding of their Fc region to the Fc γ receptor (Fc γ R) which include antibody-dependent cellular cytotoxicity (ADCC), phagocytosis and complement activation [43,44]. Ab-mediated activation of the classical complement pathway can lead to the generation of the membrane-attack complex and the lysis of virus and virus-infected cells by binding the complement protein to Fc regions of IgM, IgG1, IgG2, and IgG3. HIV has evolved defense mechanisms against Ab-dependent complement activation such as incorporating complement control proteins into its viral membrane (reviewed in [45]). This review will discuss the importance of HIV-specific ADCC and antibody-dependent cellular phagocytosis (ADCP) and not Ab-dependent complement activation.

2.2. HIV-Specific Antibody-Dependent Cellular Cytotoxicity

ADCC is a current focus of HIV research. ADCC is initiated by Fc γ R on innate immune cells recognizing and binding IgG (IgG1, IgG2 or IgG3) bound to infected cells. This flags the cell for destruction by innate immune effector cells such as natural killer (NK) cells, monocytes and neutrophils. Although Fc γ R can have both activating and inhibitory activities, HIV

protective activity is likely mediated primarily by activating Fc γ Rs such as Fc γ RI (CD64), Fc γ RIIa (CD32a), Fc γ RIIb (CD32b) and Fc γ RIIIa (CD16) (Table 1). Fc γ RI is expressed on monocytes and neutrophils and binds monomeric IgG with highest affinity and therefore, given the high concentration of serum IgG, is thought to be saturated under physiological conditions [24]. In contrast, for low affinity receptors Fc γ RIIa, Fc γ RIIb and Fc γ RIIIa, the efficient binding of monomeric IgG under physiological conditions requires the formation of immune complexes [46,47]. This is consistent with the role for such Fc γ Rs in pathogen clearance and immune-regulation [48]. HIV-specific ADCC mediated by NK cells requires aggregation (cross-linking) of Ab-bound Fc γ RIIIa [46]. Successful cross-linking of surface Fc γ Rs leads to activation of the NK cells, degranulation and secretion of both cytotoxic granules (perforin and granzyme B) and cytokines/chemokines (including IFN γ and TNF). This results in both the destruction/killing of the Ab-flagged infected cell and the amplification of the local immune response (Fig. 1C). The total amount of IgG bound to an HIV-1-infected cell is an important determinant for the effectiveness to the ADCC response [49].

3. COMMON ASSAYS FOR ADCC

The potential of ADCC responses in controlling HIV-1 has been intensively studied over the last couple of years and many different ADCC assays have been developed and

Table 1. Activating and Inhibiting Fc Receptors

FcR	CD	Receptor Type	Cell Type Expressed on	Polymorphism Influencing Ig Affinity	Order of Ig Binding Affinity	Biological Function	References
Activating Fc Receptors							
FcγRI	CD64	high affinity, binds non-complexed monomeric IgG	monocytes, macrophages, neutrophils, eosinophils, DC		IgG1, IgG3>>> IgG4>>>IgG2	phagocytosis oxidative burst cytotoxicity complement activation	[46, 48, 228, 229]
FcγRIIa-1 FcγRIIa-2	CD32a	low-affinity, aggregated IgG complexed with antigens	monocytes, macrophages, neutrophils, eosinophils, (DC)	H131 increased affinity R131 decreased affinity	IgG3>, IgG1, IgG2>>IgG4 (H131); IgG3>IgG1>> IgG2, 4 (R131)	phagocytosis oxidative burst cytokine production degranulation complement activation	[46, 48, 227-229]
FcγRIIc	CD32c	low-affinity, aggregated IgG complexed with antigens	NK cells B cells		IgG1, IgG3>> >IgG2, IgG4	phagocytosis of immune complexes and modulation of antibody production by B-cells.	[46, 48, 227]
FcγRIIIa	CD16a	low-affinity, aggregated IgG complexed with antigens	NK cells, macrophages, monocytes, eosinophils, DC	V158 increased affinity R158 decreased affinity	IgG1, IgG3>> >2, 4	ADCC and Induction of cytokine release	[46, 48, 228, 229]
FcγRIIIb	CD16b	low-affinity, aggregated IgG complexed with antigens	neutrophils, basophils		IgG3, IgG1>> >IgG2, IgG4	phagocytosis of immune complexes	[46, 48, 228, 229]
FcαR	CD89	aggregated IgA complexed with antigens	monocytes, macrophages, neutrophils, eosinophils		IgA	phagocytosis of immune complexes especially in mucosal sites	[46, 48, 230]
Inhibiting Fc Receptors							
FcγRIIb-1 FcγRIIb-2	CD32b	low-affinity, aggregated IgG complexed with antigens	macrophages, neutrophils, basophils, B cells, DC	I232 increased affinity T232 impaired recruitment to lipid raft	IgG3, IgG1> IgG4>>IgG2	regulation of B cell activation and development, development of humoral tolerance	[46, 48, 227-229]

utilized. However, all assays measure different effector functions and have some pitfalls including limited number of NK cells, donor-to-donor variability and the use of non-physiological targets (immortalized cell lines). Assay differences need to be carefully interpreted when comparing results from different studies. There is no clear consensus on the best ADCC assay to utilize that correlates most closely with improved outcomes for HIV infection since the assays described measure over-lapping functions of ADCC Abs. The assay of choice also can highly depend on the type and volume of the specimens to test.

3.1. NK Cell Activation Assay

The majority of ADCC assays use whole Env protein (gp120 or gp140), which is coated to the target cells. These target cells are mainly T cell lines where the Env protein binds to CD4 or to lectins on the surface of cells. These assays are great to study an overall response to the Env protein including conformational epitopes but lack the ability to detect and map linear epitopes. A simple whole-blood assay of cytokine expression in responses to overlapping peptides has revolutionized the study of HIV-specific ADCC

and allows for the quantification and mapping of effective epitopes through the use of linear peptides [50]. Instead of target cell lysis, Ab-mediated activation of CD3-CD56⁺ NK cells is assayed by the detection of intracellular IFN γ and CD107a (LAMP-1), a surrogate marker for NK cell activation and degranulation [51]. HIV peptides (13-15mers) mimicking HIV antigens are presented by granulocytes [52]. Using this assay breadth and characteristics of HIV-specific ADCC-dependent NK cell activation have been studied [53-58] and responses specific to Env, Pol and Vpu epitopes [59-61] have been identified. The NK cell activation assay also allows for the detection of the lytic capacity of NK cells by measuring the loss of intracellular granzyme in NK cells, which is thought to be delivered to the target cells [50].

3.2. ADCVI Assay

The Ab-dependent cell-mediated virus inhibition (ADCVI) assay tests for multiple inhibitory effects of Abs on virus growth, usually in the presence of HIV-infected CD4⁺ T cells as target cells and autologous PBMC or NK cells as effector cells. This assay can assess total HIV inhibition including classical neutralization and ADCC at the same time. It highlights the critical nature of the Fc-Fc γ R interaction. It has been shown that ADCVI activity is partially mediated by β -chemokines produced by NK cells activated through the binding of Abs or antigen-Ab-complexes due to their competition with HIV for the CCR5 co-receptor [62]. This assay has been used by many groups to assess viral inhibition [24,63,64]. However, using the ADCVI assay it is difficult to dissect the contributions of neutralization and ADCC on the overall virus inhibition. One possibility to determine ADCC activity in the ADCVI assay would be the simultaneous measurement of NK cell activation and direct delivery of granzyme B.

3.3. Luciferase ADCVI-Type Assay

Primary cells as target and effector cells have the disadvantage of limited availability and donor-to-donor variability. This limits the majority of the assay to be used in clinical trial. Alpert and team have therefore adapted the ADCVI assay using cell lines. This assay was employed to assess the ADCC responses reported for the RV144 trial [65]. The new ADCVI assay uses an NK cell line, which has been transformed to express either the human or macaque high affinity variant of Fc γ RIIIb (CD16) and a CD4⁺ T cell line that expresses luciferase from a Tat-inducible promoter (CEM.NKr.CCR5.Luc) upon HIV or SIV infection. The loss of luciferase activity is dependent upon Ab concentrations indicating the killing of virus-infected cells by ADCC [66]. The use of cell lines in combination with luciferase as an indicator of cytotoxicity enables large-scale assays on a routine basis. They also found that there is incomplete overlap between the Abs that directs ADCC against virus-infected cells versus Abs measured in neutralization and gp120-binding assays. This assay is up to two orders of magnitude more sensitive than optimized assays for neutralization using the same viruses and same plasma samples and is therefore suitable to measure ADCC when neutralization is undetectable. While this assay is great for measuring ADCC capability of Abs it does not recognize the

behavior of the Abs in the context of NK cells with different receptor/ligand combinations.

3.4. Granzyme B ADCVI-Type Assay

Another new assay is the fluorometric granzyme B cytotoxicity assay [49,67]. This assay is based on a cell-permeable fluorogenic peptide and a substrate, which generates a fluorescent signal when it gets hydrolyzed by granzyme B. This allows identification of individual target cells that have received granzyme B from the effector cells. This assay can use healthy primary CD4⁺ T cells or T cell lines, like CEM.NKr.CCR5, that are infected or coated with Env protein before autologous NK cells, serum or purified Abs and granzyme B substrate (GranToxiLux) are added. After a short incubation granzyme B positive cells are determined by flow cytometry. Further, the reduction of infected target cells can be assessed by staining for intracellular p24 expression. This assay can be performed in 96 well plates and is ideal for high-throughput. Using this assay Smalls-Mantey *et al.* did not detect a correlation of viral load or CD4⁺ T cell count with ADCC titers neither for progressors nor for long-term slow progressors. Further, ADCC activity was mainly directed against Env and, showed cross-subtype reactivity and strongly correlated with total and IgG1 binding to the surface of infected cells [49].

3.5. Chromium Release Assay

Early studies that measured ADCC responses to HIV-1 used the ⁵¹Chromium release assay [68-73] originally established to measure killing by cytotoxic T cells. In this assay target cell lines are coated with Env protein or HIV virus and labeled with the radioactive ⁵¹Chromium. Ab-mediated target cell killing by PBMC is measured by the release of ⁵¹Chromium into the cell culture supernatant. This assay was previously used to determine ADCC responses in the RV135 study [74]. The FATAL assay, a further improvement of the Chromium release assay, was developed by Sheehy and colleagues using fluorescent, and not radioactive labeling, to measure T cell specific lysis [75].

3.6. RFADCC Assay

Based on the FATAL assay, Robert-Guroff and colleagues [26] developed the rapid and fluorometric Ab-dependent cellular cytotoxicity (RFADCC) assay. The target cells used in the RFADCC assay are CEM.NKr.CCR5 cells, a CD4⁺ T cell line resistant to NK cell killing mediated by natural cytotoxicity receptors, that are coated with HIV or SIV Env protein. To assess anti-HIV ADCC killing mediated by HIV-specific IgG the Env-coated CEM.NKr.CCR5 are double labeled with the membrane dye PKH26 and the cytoplasmic dye CFSE and co-cultured with PBMC from a healthy, HIV-uninfected subject in the presence of defined amounts of the test Abs. The killing by PBMC is defined by the loss of CFSE but the retention of PKH26 resulting in the emergence of a PKH26+CFSE- population within the PKH26+ gate. The RFADCC has a versatile use: (a) frozen and healthy effector PBMCs, (b) infected or Env protein coated target cells, (c) human or macaque sera and (d) sera from infected and vaccinated individuals can be used to assess ADCC. The RFADCC assay has been widely used to

correlate vaccine-induced ADCC with protection in macaques [60,76-79]. A slight modification of the RFADCC assay employs EGFP expressing CEM.NKr.CCR5 target cells rather than CFSE staining and staining for cell death using propidium iodine (PI) [80]. This allows assessment of killed target cells by gating on EGFP-PI+ cells.

3.7. What Does RFADCC Assay Measure?

We recently demonstrated that NK cell activity is not the major killing mechanism detected in the RFADCC assay. Our data indicates that the RFADCC assay rather measures IgG-mediated monocyte function. The PKH26+CFSE- cell population typically reported as "killed" target cells in this assay are mainly CD3-CD14+ monocytes with internalized PKH26 membrane fragments, rather than CEM.NKr.CCR5 target cells that have lost CFSE [81]. Moreover, microscopy studies showed that monocytes take up PKH26-stained target cell membrane without evidence of classical phagocytosis of the intact target cell. Further experiments are required to elucidate the precise mechanisms of how the target cells are killed during this process. It is possible that the CEM.NKr.CCR5 cell line coated with gp140 Env protein may present antigen to Abs differently compared to HIV-infected or coated primary cells. This may in turn result in more efficient activation of monocytes rather than NK cells. Nonetheless, we can not rule out that the observed monocyte activity overlaps with NK cell-mediated ADCC. The RFADCC assay is definitely a useful and rapid screening assay for functional HIV-specific Abs.

4. ADCC IN HUMANS AND NON-HUMAN PRIMATE MODELS IN THE CONTEXT OF INFECTION AND VACCINATION

4.1. Role for ADCC in Controlling HIV Infection in Humans

Considerable evidence now supports the role for ADCC activity in the control of HIV infection. In HIV-infected individuals the presence of ADCC correlates with a better disease prognosis [68,82,83] potentially due to the reduction in viral load by inhibiting cell-to-cell spread and limiting the pool of infected cells. Levels of HIV-specific ADCC Abs are also correlated with slow progression of HIV infection [44,56,61,83-91]. ADCC-mediating Abs in breast milk have been reported to reduce the risk of HIV transmission to infants [92].

Aside from lower ADCC Ab titers, the level of ADCC in progressive HIV infection is probably influenced by disease-associated NK cell dysfunction. Several NK cell phenotypic alterations and subset redistributions occur during chronic (progressive) HIV infection, which could render NK cells incapable of mediating strong ADCC responses. Progressive HIV infection is characterized by expansion of a hypofunctional NK cell subset, identified as CD16+CD56-, and a decrease of the highly cytotoxic CD56dimCD16+ NK cell subset, which is not observed in uninfected or slow progressor individuals [93-95]. Furthermore, NK cells from individuals with progressive infections are more likely to exhibit increased expression of inhibitory NK cell receptors and reduced expression of activating NK cell receptors than NK cells from uninfected or slow progressor individuals (reviewed in [93]). As NK cell activation is determined by

the cumulative signal received through the unique constellation of activating and inhibitory receptors on the NK cell surface [96], the increased inhibitory, and decreased activating, surface receptor content on NK cells from individuals with progressive HIV-infection should render their NK cells less likely to be activated upon exposure to putative target cells. These alterations in NK cells in progressive infection have been shown to partially reverse after combination antiretroviral therapy (cART) induced viral suppression, and result in improved NK cell functionality [94]. Although, NK cell function is improved by cART it is not completely restored, likely due to residual ongoing immune activation [97].

4.2. ADCC at Mucosal Surfaces of the Genital Tract

In contrast to other mucosal sites that contain mostly locally produced secretory IgA, semen and cervico vaginal fluid contain more IgG than IgA and levels in the female genital tract are highly variable and dependent on hormonal regulation at different stages of the menstrual cycle [98-100]. However, little is known about the role of mucosal NK cells and monocytes preventing primary infection through mechanisms such as ADCC. ADCC-mediating Abs are found in cervico-vaginal lavages of HIV-1 infected women and are associated with significantly lower genital HIV-1 RNA [69,73]. This is of importance as the majority of HIV infections occur *via* sexual transmission across a mucosal barrier. Surprisingly, HIV-specific IgG has also been found in cervico-vaginal secretions of highly exposed seronegative individuals [101], raising the question if they have an immunological advantage [102]. While Ab-mediated effector function in the mucosa may be relevant it is difficult to predict the effect of IgG-mediated ADCC in the vaginal mucosa without knowing the IgG concentrations required *in vivo*. Effector cells capable of mediating ADCC are present in the submucosal sites and it is notable that the passive Ab transfer studies in macaques suggest a role for ADCC in preventing mucosal SHIV challenge [41]. Further studies need to define how ADCC Abs are able to diffuse into the submucosa and where effector cells like, NK cells, originate from. Another previously reported Ab-mediated immune function is facilitated by the Fc region of IgA, which binds to mucus. This bound IgA results in trapping the virus and prevents HIV infection [103,104] (Fig. 1B).

4.3. ADCC Induced by Human Vaccines - The RV144 Vaccine Trial

Previous HIV vaccine trials, which were based on inducing bnAbs or CTLs, were not efficacious [35,105]. However the recent Thailand RV144 vaccine trial showed a modest but significant protective immunity (31%, $p=0.04$) [37,106]. The immune response in this trial was primed by multiple injections of the recombinant canarypox vector ALVAC-HIV expressing HIV-1 gp120 (subtype CRF01_AE), the transmembrane-anchoring portion of gp41 from HIV-1 (subtype B) and HIV-1 Gag and Protease. This was followed by a booster injection with the same vector together with gp120 protein (AIDSVAX subtype B/E). The RV144 vaccine did not induce bnAbs or CTLs, but robust HIV-specific ADCC responses were detected [65]. This is in agreement with results from a previous vaccine trial in Thailand in 2005 (RV135) where the combination of

ALVAC-HIV and gp120 AIDSVAX B/E vaccine, but not ALVAC-HIV alone, elicited potent HIV-specific ADCC in 96% of vaccine recipients [74].

Non-neutralizing IgG Abs to the constant region 1 (C1) and variable loops 1 and 2 (V1 and V2) correlated inversely with the risk of HIV infection [65]. Post hoc analyses demonstrated that breakthrough viruses of individuals that became infected had escape mutations within the V1/V2 sequences further suggesting a protective role for immune responses to this region [107]. Additional analyses showed that participants with low serum IgA but high IgG levels with ADCC-activity had a reduced risk of infection [37,108]. Furthermore, binding of plasma monomeric IgA to Env correlated directly with the risk of infection potentially due to competition of IgA with IgG binding [65]. There is only limited evidence that IgA can mediate ADCC through binding to their Fc α R (CD89), expressed on monocytes, macrophages, neutrophils and eosinophils [46]. As such it was postulated that binding of serum IgA instead of IgG to Env would have led to abrogation of the protective ADCC activity. This was confirmed most recently by Tomaras and colleagues who demonstrated that Env IgA serum Abs elicited by the vaccine can actually interfere with binding and functional activity of Env IgG serum Abs [109]. Thus, although the elicitation of mucosal IgA responses has been a high priority for candidate HIV vaccines that focus on eliciting neutralizing Abs, the elicitation of serum IgA could have detrimental influence for IgG and Fc-mediated protection. This competition of IgA with IgG has also been shown for other diseases [110-112]. Further, HIV-specific Ab levels against the V2 loop declined over time potentially impacting on protection [113]. Although more work remains to be done, these results do suggest that ADCC is a likely immune correlate for protection in the RV144 trial [65,109]. Results also imply that in new vaccine trials it will be important to measure serum IgA/IgG ratios of Abs elicited to potentially protective epitopes.

The majority of ADCC-mediating monoclonal Abs (mAbs) isolated from vaccinated individuals of the RV144 trial target distinct, but overlapping, epitopes of the CD4 binding site in the N-terminal C1 region of gp120 and have high ADCC activity [114]. Other ADCC linear epitopes targeted the V2 region (CH58-like, CH59, HG107, HG120-like mAbs) [114,115] and the V3 region of Env [114]. Much lower levels of Ab maturation is required to produce ADCC-mediating Abs compared to bnAbs. Mutation rates for ADCC mAbs were low (0.5-5.1%) although a higher degree of VH somatic mutation correlated with ADCC activity [114]. Based on the immune correlates of the RV144 trial, future vaccines should target IgG specific to the V1/V2 region and ADCC-mediating IgG to the C1 region. This should be done in concert with reducing IgA masking serum Abs if feasible. Nonetheless, these results do not exclude an important role for bnAbs or robust and broad CTLs as vaccine targets, although they have been difficult to induce to date [35].

4.4. ADCC in Non-Human Primate Models

Studies of acute and chronic SIV_{mac251} infected rhesus macaques demonstrate that ADCC responses are detectable as early as 3 weeks after infection; the magnitude of the ADCC response is inversely associated with plasma viral

RNA levels [116] and ADCC responses persist through the course of infection [117]. Furthermore, sustained ADCC in SIV-infected macaques has been correlated with delayed disease progression [118]. Vaccination studies in macaques typically elicited multiple responses including CTL, nAbs, and ADCC. It is therefore difficult to definitely ascribe a role for ADCC in the protection observed. Nonetheless, studies suggest that vaccine-elicited ADCC Abs played at least a partial role in protection and/or correlated with reduced peak viremia [76,78,119-121]. Using a live-attenuated SIV Δ Nef vaccine ADCC responses developed over time and were dependent upon the persistent low replication of the vaccine strain. The magnitude of ADCC response was associated with complete protection against SIV_{mac251} challenge [91]. Although this approach will never reach the human clinical phase due to safety concerns, it demonstrates the importance of persistent stimulation of the Ab responses. The recent study by Xiao *et al.* 2012 showed that a replicatingAd5hr-HIV/SIV vaccine administered by different mucosal routes elicited secretory IgA at multiple mucosal sites. Interestingly the reduction in viremia correlated inversely with ADCC titers pre-challenge and ADCC titers correlated with the number of challenges required for infection [122]. Several new promising vaccine candidates that induce a broader and more robust HIV-specific ADCC are now being studied in macaques and include gp41 subunit virosomes [123] and a DNA prime-boost regimen using a cDNA encoding a chimeric HIV protein (Tat-Env-Rev) [124]. It was previously shown that ADCC Abs are mainly elicited against conformational epitopes and ADCC is mainly mediated by IgG subclass 1 in macaques [125]. However, several studies now show that ADCC Abs to V1 and V2 and non-neutralizing Ab titers arise as early as 2 weeks post vaccination and correlate with prevention of infection [124,126]. These studies also highlight the importance of including Env as antigen to achieve protection mediated by ADCC Abs. However, so far there is no direct evidence that mAbs with ADCC activity but not neutralizing activity can afford protection from infection in a non-human primate challenge model [29,33]. The ADCC-mediating mAbs isolated from the RV144 human clinical trial [109,114] now offers a possibility to test these new Abs in non-human primate passive immunization protection studies. This will provide crucial evidence if passive transfer of ADCC-mediating mAbs can provide protection similar to neutralizing Abs.

4.5. ADCC Abs and Neutralizing Abs - Their Role in Forcing Escape

Mutational escape to avoid potent anti-HIV immune responses is a hallmark of HIV infection of humans. HIV has an error-prone reverse transcriptase that results in the ready generation of variants that may have a selective advantage by being less prone to immune recognition. Escape was first described for CD8⁺ CTL responses [127] and subsequently for bnAb responses [128-131]. Escape likely also occurs at particular CD4⁺ T helper responses although this seems less common [132]. The selection of escape mutants implies that immune responses are imparting significant pressure on virus replication. Escape strategies from bnAb include heavy glycosylation of Env and conformational masking of receptor binding sites [133-135]. Recently, we described mutational

escape from HIV-specific ADCC responses [60]. By mapping linear ADCC epitopes from subjects with chronic HIV infection and then sequencing the subject's autologous virus across the epitope, we found that in around 2/3 of the epitopes mapped, the subject's ADCC response was no longer able to recognize their autologous virus. In subjects that were studied over time, ADCC escape appeared to occur relatively early in infection. Interestingly, Env-specific ADCC epitopes that escaped were common in the C1 region of Env, a site commonly targeted by ADCC Abs in the RV144 vaccine trial. We speculate that pre-existing ADCC responses prior to infection may effectively limit active viral replication and thereby limit opportunities for immune escape. Env is highly diverse and is able to mutate readily without apparent significant fitness costs [136-139]. This may limit the utility of ADCC responses in controlling established infection. ADCC responses to more conserved viral proteins may result in larger fitness costs to the virus if/when escape occurs. We recently observed ADCC responses to Pol and smaller viral proteins such as Vpu [50,57,59,140]. Vpu-specific ADCC responses were associated with slow HIV progression [61]. Others have described ADCC responses to Tat [120] and Nef [72]. However, it is unclear how ADCC responses to non-cell-surface HIV proteins would assist in targeting HIV infected cells. We speculate that ADCC recognition of viral debris on bystander cells may mark a site of recent active infection and potentially recruit effective immune responses (*via* release of cytokines/chemokines) that may limit local replication. Nonetheless, we or others have not yet observed convincing immune escape at non-Env ADCC epitopes. This suggests either that these responses are relatively ineffective or that fitness costs may more effectively constrain the generation of escape mutants at non-Env ADCC epitopes.

5. OTHER FACTORS THAT ARE IMPORTANT FOR ADCC

Optimizing anti-viral ADCC responses will also require a detailed understanding of the NK cell and exogenous features that shape ADCC effector responses. The ability of NK cells to mediate ADCC is determined by a cluster of factors (Fig. 2), including (1) the developmental /ontological process of NK cell education [141-144], (2) the phenotype of NK cell surface receptors expressed [145], (3) polymorphisms in Fc γ RIIIa [146,147], (4) exogenous soluble factors, such as cytokines [148] as well as (5) Fc glycosylation [149] that can sway NK cell functionality.

5.1. Importance of NK Cell Education for Effective ADCC Responses

The ability of NK cells to mediate ADCC or natural cytotoxicity upon encountering putative target cells is determined by an ongoing ontological process known as NK cell education or licensing. This developmental pathway, which endures for the duration of the NK cell lifespan, involves the continuous interaction of the activating and inhibitory receptors on the NK cell surface with constitutively expressed self-ligands on other cells (Table 2) [150-153]. In a manner that prevents aggressive immune responses against healthy autologous cells, the signals from these interactions are integrated and functional potential is conferred to NK cells with cumulative inhibitory signals, but not to those that receive cumulative activating signals. Several lines of evidence support the idea that this

process is a continuum and not a simple on/off process [154,155]. Instead, it appears as if the net magnitude of the inhibitory signal determines the effector function of an NK cell may mediate upon activation. It has been demonstrated that the first effector function capacity conferred upon NK cells is that of degranulation, and that with more education, intense inhibitory signaling in particular, NK cells gain the ability to produce cytokines [154].

The early research that shaped the current understanding of NK cell education was conducted on the direct Ab-independent activation of NK cells [150]. Several groups, however, have now demonstrated that the ability of NK cells to mediate ADCC is also influenced by the educational process [141-144]. Indeed, the degree of NK cell activation and target cell cytolysis observed upon the stimulation of NK cells by Ab-mediated CD16 cross-linking or by Ab-coated allogeneic transformed cells is determined by the expression of inhibitory killer cell immunoglobulin-like receptors (KIR) on NK cells, as well as if the NK cell donor also carried the major histocompatibility complex class I (MHC-I or HLA-I) ligand for the particular KIR receptor. Anfossi *et al.* confirmed that NK cells that expressed inhibitory KIR2DL receptors were more functional, as measured by degranulation and cytokine synthesis, upon Ab-mediated cross-linking of CD16 or stimulation with Ab-coated target cells if the NK cells were obtained from donors that carried the appropriate HLA-C ligand [144]. Similarly, several studies have demonstrated that stimulation of KIR3DL1 expressing NK cells with Ab-coated allogeneic target cells results in higher cytolysis of the Ab-coated target cells and higher NK cell cytokine production if the NK cells are obtained from individuals carrying HLA-B alleles that exhibit the HLA-Bw4 epitope for KIR3DL1 [141,143].

The observation that KIR3DL1/HLA-Bw4 combinations can educate NK cells for enhanced ADCC is particularly interesting with regards to HIV infection. Several epidemiological studies have demonstrated that certain allelic combinations of KIR3DL1/HLA-Bw4 can confer protection from HIV infection or progression towards AIDS in HIV-infected individuals [156-158]. The mechanism(s) of this protection, however, has not yet been determined. It has generally been hypothesized that KIR3DL1/HLA-Bw4 combinations confer protection against HIV by educating NK cells for heightened functionality against HIV-infected T cells [159]. Counter-intuitively, however, KIR3DL1 expressing NK cells from HLA-Bw4 carriers have been shown to be hypofunctional against autologous HIV-infected cells [63]. Indeed, direct co-culture of NK cells and autologous infected T cells resulted in no observable NK cell degranulation. This lack of functionality directed against autologous infected cells most likely reflects the inability of KIR3DL1 expressing NK cell to receive a strong enough cumulative activating signal upon encountering the infected autologous cell. As Abs coated on the surface of infected cells can increase NK cell activating signals through Fc γ RIIIa (CD16) ligation, the mechanism of ADCC offers an avenue to overcome this activating signal deficit. Furthermore, if NK cell education through KIR3DL1/HLA-Bw4 combinations determines the level of ADCC mediated by NK cells towards autologous cells, ADCC may offer an explanation for the protective role of carrying certain KIR3DL1/HLA-Bw4 allelic combinations.

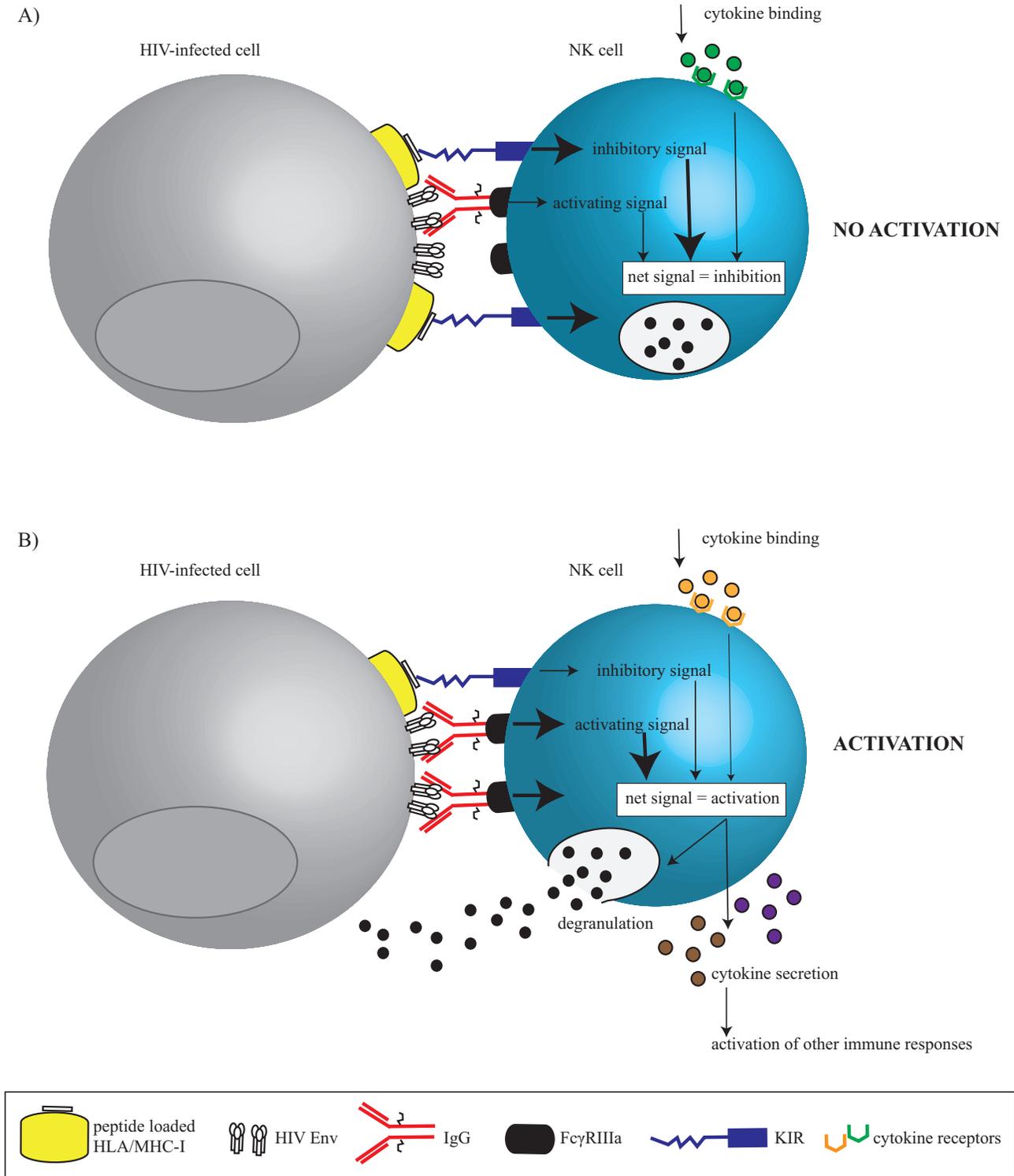


Fig. (2). The fine balance of NK cell activation and ADCC function. **(A)** No NK cell activation: The magnitude of the activating signal received through the antibody-FcγRIIIa interaction is lower than the inhibitory signals through the KIRs resulting in an overall inhibitory signal. Some cytokines including TGF-β can enhance inhibition by making it more difficult to obtain a cumulative activating signal. **(B)** NK cell activation: The magnitude of activating signal received through the antibody-FcγRIIIa interaction is higher than the inhibitory signals through the KIRs resulting in an overall activating signal. This results in degranulation, chemokine and cytokine secretion (e.g. TNF, IFNγ, RANTES, MIP1α, MIP1β) and eventually leads to the killing of the cell and activation of other immune responses. Some cytokines including IL-15 can enhance the activation by increasing activating receptor expression, increasing perforin synthesis or/and enhancing NK cell education.

Table 2. Activating and Inhibitory NK Cell Receptors

Receptor	Ligand	Effect on Education	Effect of HIV	References
Activating Receptor				
NKp30	B7-H6 PP65 PfEMP1 Viral HA Heparin or heparin sulfate	unknown physiological role	Decreased expression	[231]
NKp44	Viral HA Heparin or heparin sulfate Sialylated and sulfated proteoglycans PCNA	unknown physiological role	Decreased expression	[231]
NKp46	Viral HA Heparin or heparin sulfate Unknown tumor ligands	unknown physiological role	Decreased expression	[231]
Activating KIR (e.g., KIR2DS1 and KIR3DS1)	Classical HLA-I	Decreases in presence of ligand	Can be decreased, increased or unaltered in expression	153, 232-234
NKG2C	HLA-E	unknown physiological role	Increased	[165, 235]
NKG2D	MICA MICB ULBPs	unknown physiological role	Can be decreased or unaltered in expression	[236, 237]
Inhibitory Receptor				
Inhibitory KIR (e.g., KIR2DL1 and KIR3DL1)	Classical HLA-I	Enhances in presence of ligand	Can be decreased, increased or unaltered in expression	[143, 144, 151, 232-234, 236, 237]
NKG2A	HLA-E	Enhances in presence of ligand	Decrease in general, but increased expression on cytolytic subset	[165, 235, 236, 238, 239]

B7-H6: glycosylated member of B7 co-stimulatory proteins - acts as NKp46 ligand on tumor cells; PP65: human cytomegalovirus tegument protein; PfEMP1: Plasmodium falciparum erythrocyte membrane protein 1; viral HA: hemagglutinin; PCNA: proliferating cell nuclear antigen (tumor antigen); MICA, MICB: MHC class I polypeptide-related sequences A and B; ULBPs: unique long 16-binding peptide.

In order for educated KIR3DL1+ NK cells to mediate ADCC against autologous targets it is necessary that the activating signal received through CD16 reach a higher level than inhibitory signals received by the NK cell *via* KIR3DL1/HLA-Bw4 interactions. A recent investigation of the role of NK cell education in mAb-induced ADCC against malignant target cells demonstrated that KIR3DL1/HLA-Bw4 interactions were sufficient to inhibit the functionally advantaged “educated” NK cell subset from mediating robust anti-tumor ADCC [141]. Studies in our own laboratory, however, have demonstrated that this may not be the case for anti-HIV ADCC. Indeed, when autologous cells were coated with HIV peptides and labeled with anti-HIV Abs, we observed higher Ab-dependent NK cell activation in the KIR3DL1+ than the KIR3DL1- subset of NK cells from HLA-Bw4 carriers [142]. Furthermore, the NK cell activation observed in the KIR3DL1+ NK cell subset was higher in the HLA-Bw4 carriers than in individuals with no HLA-Bw4-containing alleles. Although the reason for the divergent observations between the anti-tumor and anti-HIV ADCC literatures are unknown, it is feasible that the utilization of polyclonal anti-HIV Abs allowed a greater density of Abs to accumulate on the HIV ADCC target cells than was obtained with the mAb anti-tumor therapy. This interpretation would be consistent with the study by Smalls

Mantey *et al.* showing that the degree of anti-HIV ADCC is correlated with the density of cell-bound IgG [49].

Cumulatively, there is a strong body of evidence that the ontological process of NK cell education influences NK cell-mediated ADCC [141-144]. Education of NK cells through KIR3DL1/HLA-Bw4 combinations confers enhanced ADCC function, which may partially explain how certain allelic combinations of this inhibitory receptor/ligand combination protects from HIV disease progression [156,157]. Perhaps the most important implication of the entanglement of anti-HIV ADCC and NK cell education is the ramifications for HIV vaccine development. If vaccines are designed to induce ADCC Abs, they may be less effective in individuals with poor NK cell education.

5.2. Cumulative Signals Govern NK Cell Responsiveness to ADCC Abs

NK cell education merely represents the first level of NK cell regulation. After the functional potential of an NK cell is determined through the education process, the activation of an NK cell upon encountering a putative target cell is determined by the cumulative signal received through the interaction of the NK cell’s inhibitory and activating receptors with the ligands

present on the target cell (reviewed in [96]). HIV infection of CD4⁺ T cells has been demonstrated to induce several alterations in the cellular phenotype that increases the resistance of the CD4⁺ T cell to direct or Ab-dependent lysis by NK cells (reviewed by [160]).

Immediately following the infection of CD4⁺ T cells, the HIV Nef protein downregulates HLA-A and B from the cell surface [161,162]. This makes the infected cell less susceptible to attack from cytotoxic T cells. But because HLA-A and B serve also as ligands for inhibitory NK cell receptors it also increases the chances that an NK cell could lyse the cell. To skirt this possibility HIV has developed alternative mechanisms to evade NK cell attack. For example, HIV-infected cells exhibit increased HLA-E on their cell surface, which is due to the stabilization of HLA-E by a peptide from the p24 protein (Gag) [163,164]. HLA-E serves as the ligand for the inhibitory NK cell receptor NKG2A [165]. Furthermore, HIV-infected cells have been demonstrated to maintain the constitutively expressed level of HLA-C, which serves as a ligand for the NK cell inhibitory KIR2DL receptors [161]. The increased HLA-E expression and maintenance of HLA-C expression on HIV-infected targets serve to decrease NK cell activation upon direct and Ab-dependent interactions and are effective NK cell evasion strategies of HIV [145,166].

Aside from regulating the expression of ligands for inhibitory NK cell receptors, HIV-infected cells have also been shown to negatively regulate the expression of ligands for activating receptors on the infected cell's surface. Several studies have observed a decrease in the expression of ligands for the co-activating NK cell receptor NK-T and -B antigen (NTB-A) [167] and activating receptor NKp44 [168]. These mechanisms have been demonstrated to decrease the ability of the NK cell to directly attack the infected T cell. Although not formally demonstrated, as activating receptors can act in conjunction with CD16 [169], it is foreseeable that these alterations in ligands for activating receptors may also decrease the ability of NK cells to mediate anti-HIV ADCC.

Collectively, regulation of ligand expression on HIV-infected target cells illustrates the mechanism that HIV has developed to evade NK cell activity. These observations, however, should not be viewed as discouraging, as experimental modifications of these systems have demonstrated that with a correct balance in activating and inhibitory signals HIV-infected lymphocytes are targetable [49,145]. This observation makes ADCC a particularly attractive immune response, as polyclonal Ab responses can be utilized to increase the cumulative activating signal.

5.3. FcγRIIIa Polymorphisms and Altered Signaling

An important factor that determines the ability of an NK cell to mediate ADCC through FcγRIIIa is genetic polymorphisms influencing the receptor's affinity for cell-bound IgG. One important FcγRIIIa polymorphism that has been demonstrated to have clinical ramifications is the presence of a valine (V) or phenylalanine (F) at position 158 [146]. The V158 polymorphism confers the receptor with a higher affinity for IgG; whereas, the F158 polymorphism results in a receptor with a lower affinity for IgG. Far from being an interesting laboratory observation, it has been

shown that individuals with FcγRIIIa-V158 polymorphism have more successful outcomes than those with FcγRIIIa-F158 upon commencement of mAb therapies of malignancies [147]. The relevance of these polymorphisms for anti-HIV ADCC, however, is not clear. Studies of vaccinated subjects in the VAX004 trial has revealed that low-risk individuals carrying the V158 polymorphism had a higher risk of HIV infection than low-risk individuals without the polymorphism [170]. Furthermore, carriage of V158 in low-risk subjects resulted in them being as susceptible to HIV infection as high-risk vaccinees, regardless of the high-risk subject's 158 polymorphism. Similarly, when individuals vaccinated during the RV144 trial were analyzed post infection, it was revealed that individuals carrying the V158 polymorphisms exhibited more rapid CD4⁺ T cell decline and quicker time to HAART initiation [171]. Studies in non-vaccinated individuals with HIV infection have been less conclusive. For example, one recent study was unable to demonstrate the polymorphism at position 158 as being an important determinant of anti-HIV ADCC [87].

An additional aspect of FcγRIIIa biology that appears to be important for determining the degree of NK cell activation observed upon triggering of the receptor is the intracellular signaling pathway utilized [172]. Due to a positive charge in its transmembrane region, the FcγRIIIa receptor has the ability to associate with the CD3ζ and FcεRIγ chain adaptor proteins in homo and heterodimer formats, which is essential for activating signaling capacity. It has recently been shown that some healthy controls carry a subset of NK cells that lack FcεRIγ chain expression. In this NK cell subset FcγRIIIa associates only with CD3ζ. These NK cells mediate higher degranulation and cytokine synthesis upon activation through FcγRIIIa. Furthermore, the authors have demonstrated that this NK cell subset has lower expression of several inhibitory KIR, suggesting that the mediation of ADCC by these NK cells is not as restricted by NK cell education as FcεRIγ chain expressing NK cells.

5.4. Influence of Cytokines and Other Exogenous Soluble Factors on NK Cell-Mediated ADCC

In addition to factors intrinsic to the NK cell, the level of NK cell activation and cytolysis observed upon interaction with ADCC target cells can also be influenced by exogenous soluble factors, such as cytokines. A large body of literature demonstrates that cytokines, acting alone or synergistically, can have either enhancing or inhibiting effects on NK cell functionality upon direct and Ab-dependent stimulation. For example, interleukins (IL) 2, 10, 12, and 15 have been demonstrated to enhance the functional output of NK cells after stimulation; whereas, IL-4 and TGF-β have been shown to decrease NK cell functional output after stimulation [148,173-176]. The mechanisms exerted by cytokines to influence NK cell functionality are not clearly delineated. Mechanisms of cytokine-mediated enhancement of NK cell functionality include increased activating receptor expression [177,178] and increased perforin and granzyme production [179]. Interestingly, however, it has been demonstrated that cytokines which enhance NK cell functionality may exert their influence *via* utilization of the NK cell education pathway. Juelke *et al.* showed that treatment of NK cells with IL-15 led to

simultaneous increases in KIR expression and NK cell functionality [180]. Furthermore, it was demonstrated that the NK cells exhibiting increased functionality were the same NK cells that up-regulated inhibitory KIRs for which the NK cell donors carried HLA-I ligands.

Further research is required to elucidate the mechanism *via* which cytokines influence NK cell functionality. With proper understanding cytokines may be able to be utilized to design more successful anti-viral immunotherapies or vaccines.

5.5. Influence of Fc Glycosylation Patterns on IgG Effector Function

Protective Ab-mediated Fc effector function requires efficient interaction between the Fc fragment of the IgG and the Fc γ R. However, that binding is not only dependent on the IgG isotype or the Fc γ R type and its affinity, but also depends on the specific Fc glycan profile of the IgG [149]. The IgG Fc region contains a single consensus glycosylation structure also known as core heptasaccharide, at the unique N-glycosylation site (N297, asparagine 297) in the CH2 domain of each IgG heavy chain. This conserved core glycosylation consists of two N-acetylglucosamine and three mannose residues. Optional, variable residues of a core fucose, N-acetylglucosamine, galactose and sialic acid can be found attached to the heptasaccharide core (reviewed in [181,182]). It has been shown that Fc glycosylation is altered during inflammation [183] or during HIV infection [184]. Furthermore, different glycoforms have been shown to impact on the Ab-effector function including ADCC. For example, Abs that are sialylated demonstrate a reduced Fc γ R affinity and are less efficient in mediating ADCC [185]. In contrast, Abs that lack core fucosylation display increased affinity to Fc γ RIIIa and increased ADCC [186]. Although non-fucosylated Abs have been mainly used in studies assessing their therapeutic potential against cancer (reviewed in [181,187,188]), HIV ADCC-mediating mAbs b12 and 2G12 with altered glycosylation pattern also displayed improved Ab-mediated effector function. 2G12 variants that lacked the core fucose demonstrated better binding to Fc γ RIIIa and mediated higher HIV inhibition [189]. Similar, the non-fucosylated b12 variant showed improved ADCC although it did not enhance ADCC activity *in vivo* [190]. These results emphasize that the Fc glycosylation pattern plays a key role in Ab function and modification might improve anti-HIV potential and should be considered in future vaccine studies.

6. INTEREST IN MONOCYTES AS MEDIATORS OF AB EFFECTOR FUNCTIONS

Most viruses do not directly activate complement or bind to phagocytic cells like macrophages, monocytes, dendritic cells (DC), eosinophils and neutrophils [48]. Endocytosis (or macropinocytosis) rather than phagocytosis occurs if the target is small. This process involves less dramatic changes in the acceptor cell membrane. Ab-dependent phagocytosis is the process of internalization of the entire, mainly large (>1 μ m), opsonized target by the phagocytic effector cell. Phagocytosis is initiated by the interaction between an Ab-

opsonized target through the Fc fragment of the IgG and the Fc γ R. Steps involved in phagocytosis include close cell-cell contact, pseudopod formation of the effector cell and eventually full engulfment of the target cell. This results in clearance of the target cell, immune complexes, and possible antigen presentation by the phagocytic effector cells to initiate other immune responses (Fig. 1D). During both endocytosis and phagocytosis effector cells internalize the entire target [191]. The phagocytic immune function is strongly modulated by the Fc γ R type and the Fc domain of the IgG subclasses 1 to 4. Competition between pre-bound and free serum IgG has been described as a major reason for the high IgG concentration required for eliciting phagocytosis *in vivo* probably due to different binding avidity of free and multimerized IgG [192]. This highlights the complexity of Fc domain-driven effector functions: the binding of the variable domain (Fab) of the IgG to the antigen and the binding of the Fc domain to each Fc γ R. There has been an increasing interest in HIV-specific Ab-dependent cellular phagocytosis (ADCP) in the recent years. ADCP is an important connection between the adaptive and innate immune system, by both priming the adaptive immune response as well as in the clearance of virus. ADCP may not only rapidly remove HIV or HIV-infected cells from the circulation, but could also affect immune complex-induced inflammation, implicated in driving disease.

6.1. ADCP in HIV

Impaired phagocytic function of monocytes and macrophages in HIV infection has been extensively described and is caused by (a) the loss of Fc γ R intracellular signaling molecules and (b) the dysregulation of actin polymerization which plays a critical role in pseudopod-formation and ingestion of phagocytosed particles (reviewed by [193]). Further, alterations in Toll-like receptor signaling and modulation of apoptotic responses have been reported to play a role in monocyte dysfunction (reviewed in [194]). More recent studies have reported alterations in Fc γ R expression, which also likely contributes to the impairment in ADCP during HIV infection [195]. A new ADCP high-throughput assay was developed by Ackerman and colleagues [196,197] and now allows for the assessment of the phagocytic potential of Abs at different stages of HIV infection. The assay employs Env protein coated fluorescent beads that are opsonized with serum or purified IgG and subsequently incubated with the monocytic cell line THP1, which expresses all 3 main Fc γ Rs. The assay can assess Ab-mediated phagocytosis, Ab-induced cytokine secretion by monocytes (including IL-10, IL-8, IFN γ and TNF), and patterns of Fc γ Rs usage by the addition of Fc γ R blocking Abs. Dugast *et al.* found a robust increase in Fc γ RI expression on monocytes and myeloid DCs in acute HIV infection. In contrast Fc γ RII and Fc γ RIIIa expression is significantly down-regulated on phagocytic cells in both chronic and untreated HIV infection [195]. Differential expression of Fc γ Rs is associated with the loss in the ability to respond to Ab-opsonized targets, potentially contributing to a failure in viral clearance in progressive HIV infection. This is in agreement with irreversibly impaired ADCC function of NK cells with reduced Fc γ RIIIa (CD16) expression in chronic HIV infection [198].

6.2. The Role of Fc γ R2 (CD32) in HIV

Fc γ R2 has 5 isoforms, IIA1, IIA2, IIB1, IIB2 and IIC. Fc γ R2 is the most widely expressed Fc γ R on human immune cells and is the only receptor that is able to bind IgG2, but not IgG4. Two Fc γ R2 polymorphisms (Fc γ RIIA-H131 and Fc γ RIIA-R131) lead to distinct functional differences due to their differential binding to IgG. Fc γ RIIA-H131 has a much higher affinity for complexed IgG2 than Fc γ RIIA-R131 [199]. A more efficient uptake of IgG2-HIV immune complexes by monocytes from subjects with H131 rather than R131 Fc γ R2 homozygote polymorphisms has also been shown [200]. The homozygous R131 Fc γ R2 genotype is associated with faster HIV disease progression compared to the homozygous H131 or the heterozygous RH131 genotype. Furthermore, HIV-infected patients who had higher therapeutic vaccine-induced IgG2 levels against p24 prior to the cessation of cART and “high-affinity” Fc γ R2 genotypes showed the slowest increase in plasma HIV RNA level after cessation of cART [201].

The ability of Abs from infected subjects to mediate ADCP differ in their quantity as well as quality in regard to their usage of Fc γ Rs. Abs from controllers and untreated progressors exhibit increased phagocytic activity. Further, Abs displayed alterations in Fc-domain glycosylation, and interestingly have a more profound interaction with Fc γ RIIA and Fc γ RIIB [202] which explains the increased phagocytic activity. Studies with the bnAb b12, which has been mutated in the Fc region to either increase or decrease Fc γ R2 affinity, showed that Fc γ RIIA is a powerful mediator of ADCP [24]. This is in agreement with the loss in ADCP activity induced by the selective blockade of Fc γ R2 alone [24,195], suggesting that Fc γ R2 is potentially the most important Fc γ R involved in ADCP [195]. Blocking experiments have recently shown that ADCC activity is also affected by preferential binding of IgG to Fc γ RIIA [203].

6.3. Antibody-Dependent Trogocytosis

Trogocytosis is the active exchange of plasma membrane fragments and surface molecules (like receptors) between immune cells that form an immunological synapse [204]. Although, trogocytosis was first described for CD8+ T cells [205] which capture antigen loaded MHC-I complexes from antigen-presenting cells *via* the T cell receptor, it is now known that CD4+ T cells [206,207], B cells [208], NK cells [209-211], DCs [212,213], monocytes [214,215], macrophages [216,217] and granulocytes [218] can also perform trogocytosis. Two types of trogocytosis have been described: adhesion molecule-mediated trogocytosis and Ab-dependent trogocytosis [219], which is highly dependent on the interaction between the antigen-Ab complex and the Fc γ R [218]. Trogocytosis requires close cell-cell contact between two or multiple live cells [220], occurs within minutes and involves transfer of intact proteins [221,222]. It is an active process and requires both antigen-receptor signaling together with an actin cytoskeleton and cell membrane remodeling [221]. Very little is known about the nature of the proteins that are exchanged or transferred or the fate of the cells after trogocytosis. Many proteins have been identified that are exchanged or snatched including MHC molecules, co-stimulatory molecules, adhesion molecules

(e.g. ICAM), receptors (e.g. NK cell receptor) and tumor antigens [204].

ADCC-type Abs appear capable of initiating monocyte-mediated trogocytosis and this may be an under-appreciated mechanism leading to cell killing. Ab-dependent trogocytosis was studied in the cancer field where macrophages were shown to remove the tumor antigen and the bound therapeutic mAb from opsonized cells in an Ab-FcR-dependent manner [223]. Studies of anti-cancer ADCC mAbs demonstrated the importance of Src family kinase activity, PI3 kinase activity and actin rearrangement in the process of trogocytosis [217,224]. Further, it was shown that serum or plasma factors are critically involved in the mechanism of Ab-dependent trogocytosis [215,218].

During the study of anti-HIV ADCC-mediating Abs we recently observed that THP-1 cells, a monocytic cell line, and primary monocytes obtained PKH26 labeled target cell membrane which was only observed if HIV Env-coated target cells were opsonized with HIV-specific serum/plasma Abs (Fig. 3) [81]. In agreement with our results, Beum *et al.* [191] observed the uptake of PKH26 labeled target cell membrane by the THP-1 cells, but only if target cell were opsonized with Rituximab (anti-tumor mAb). This study excluded unspecific uptake of free complexed antigen-mAb after dissociation from cells or of free PKH26 dye leaking from the donor cells into the medium. Similar to Beum *et al.* we did not observe phagocytosis of target cells by effector cells in our experiments and therefore the transfer of PKH26 labeled membrane can best be explained by trogocytosis. Interestingly, the exchange of cell membrane fragments did not kill or induce apoptosis when studied within a short time frame (<1 hour) [191,223]. During the 4-hour assays performed, we observed that HIV Env-coated target cells undergoing trogocytosis displayed fragmented nucleus morphology, an indicator for apoptosis. Horner *et al.* also observed apoptosis of target cells following cell membrane exchange during a 24-hour ADCC assay using granulocytes [225].

Although trogocytosis has been studied for some time, the mechanisms and functional consequences of these transfers remain unclear. For example, the fate of a HIV-infected target cell experiencing Ab-dependent trogocytosis is unknown. Additional work is also needed to understand how cells generate the forces needed to overcome the strong hydrophobic interaction to allow cell membranes to break up. Improved live imaging techniques may help to precisely dissect and understand the process of Ab-dependent trogocytosis and its consequences *in vivo*. Further work is needed to examine if HIV-specific trogocytosis is an important Ab-mediated immune effector function and to dissect the biological relevance in the context of HIV infection. How best to optimize induction of Ab-dependent trogocytosis for both vaccination and therapeutic purposes will require further research before it can become a recommended routine measurement of Ab function in future vaccine trials.

7. CONCLUSION

The results of the RV144 vaccine trial have reinvigorated the field of HIV vaccine research on non-neutralizing

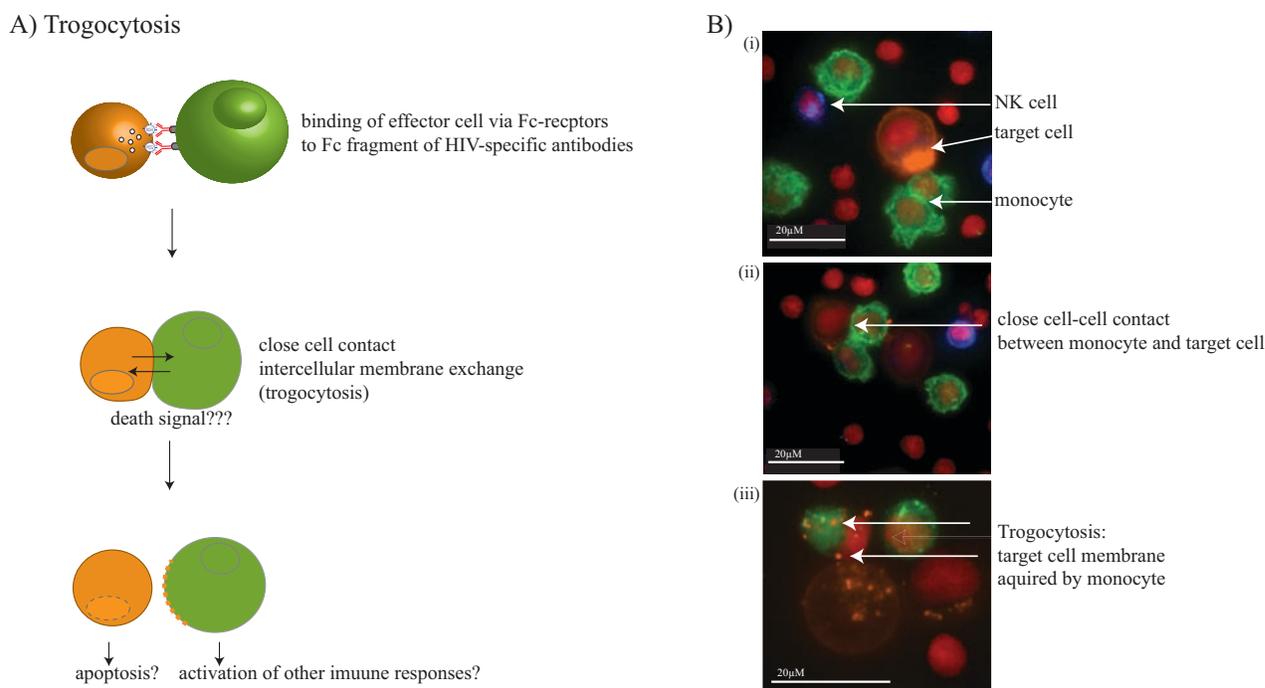


Fig. (3). Antibody-dependent Trophocytosis. **(A)** The mechanism of trophocytosis is characterized by the exchange of membranes between a target cell (orange) and an effector cell (green) which is usually phagocytic. Trophocytosis is triggered by antibody binding to the antigen and the Fc γ R with its Fab and Fc region, respectively. If the target cell receives an apoptotic signal from the effector cell during the membrane exchange is not known. **(B)** Microscopic analysis of HIV-antibody mediated trophocytosis. (i) HIV Env coated CEM.NKr.CCR5 cells (orange, target cells) in the presence of anti-HIV antibodies and primary monocytes (green) and NK cells (blue) as effector cells from a healthy blood donor (0 min); (ii) close cell-cell contact between target cell and monocytes and beginning trophocytosis (30 min); (iii) monocytes have acquired labeled target cell membrane (arrow) (90 min).

binding Abs. New investigations on the role of innate immunity in Ab-mediated effector functions and their role in preventing or controlling HIV infection are now underway [226]. This new field of HIV research has benefited from advanced, more sensitive and high-throughput assays that precisely assess ADCC and ADCP responses.

Identifying protective non-neutralizing Ab epitopes should ultimately lead to a more precise understanding of the utility of these responses in future. We speculate that some but not all ADCC Ab epitopes will be potential targets for future vaccine candidates in a similar way as for CTL and bnAb responses. The first recognition and characterization of immune escape from ADCC demonstrates the importance of this immune response in HIV infection.

The importance of contributing factors other than Ab-antigen specificity that influence an effective HIV Ab-effector function has become clear in recent years. A better understanding of the preferential binding of certain IgG subclasses for specific Fc γ R and their induced signaling capacity is needed. Fc γ R-Ab interactions are likely to influence the outcome of HIV infection and vaccination which will help to optimize HIV treatment and vaccination.

An exciting area of HIV research is the role of monocytes in mediating HIV-specific effector functions such as ADCP and trophocytosis. Understanding the relative contributions of distinct Fc γ R effector functions, such as

ADCC or ADCP, towards protective immunity will be important in rationally designing better HIV vaccines.

ABBREVIATIONS

Ab/Abs	=	Antibody/antibodies
ADCC	=	Antibody-dependent cellular cytotoxicity
ADCP	=	Antibody-dependent cellular phagocytosis
bnAb	=	Broadly neutralizing antibodies
cART	=	Combination antiretroviral therapy
Env	=	Envelope
HIV	=	Human immunodeficiency virus
KIR	=	Killer cell immunoglobulin-like receptors
mAb	=	Monoclonal antibody
FcR	=	Fc-receptor

CONFLICT OF INTEREST

The authors have no conflict of interest.

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PATIENT CONSENT

Declared none.

HUMAN/ANIMAL RIGHTS

Declared none.

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