



# Natural Killer Cells in Antibody Independent and Antibody Dependent HIV Control

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Infection with the human immunodeficiency virus (HIV), when left untreated, typically leads to disease progression towards acquired immunodeficiency syndrome. Some people living with HIV (PLWH) control their virus to levels below the limit of detection of standard viral load assays, without treatment. As such, they represent examples of a functional HIV cure. These individuals, called Elite Controllers (ECs), are rare, making up <1% of PLWH. Genome wide association studies mapped genes in the major histocompatibility complex (MHC) class I region as important in HIV control. ECs have potent virus specific CD8<sup>+</sup> T cell responses often restricted by protective MHC class I antigens. Natural Killer (NK) cells are innate immune cells whose activation state depends on the integration of activating and inhibitory signals arising from cell surface receptors interacting with their ligands on neighboring cells. Inhibitory NK cell receptors also use a subset of MHC class I antigens as ligands. This interaction educates NK cells, priming them to respond to HIV infected cell with reduced MHC class I antigen expression levels. NK cells can also be activated through the crosslinking of the activating NK cell receptor, CD16, which binds the fragment crystallizable portion of immunoglobulin G. This mode of activation confers NK cells with specificity to HIV infected cells when the antigen binding portion of CD16 bound immunoglobulin G recognizes HIV Envelope on infected cells. Here, we review the role of NK cells in antibody independent and antibody dependent HIV control.

**Keywords:** HIV, elite controllers, NK cells, killer immunoglobulin-like receptors, HLA, ADCC, antibody dependent NK cell activation

## INTRODUCTION

Natural Killer (NK) cells are innate immune cells with anti-viral and anti-tumor activity (1). NK cells detect and respond to neighboring cells that lack “self” human leukocyte antigens (HLA). This response pattern arises from a process called education that involves the interaction of inhibitory NK cell receptors (NKR) with their self HLA ligands (2–4). Educated NK cells respond to virus-infected, stressed and damaged cells that appear to NK cells as “missing self” due to their having a

reduced expression of cell surface HLA. Educated NK cells are tolerant to self-cells expressing sufficient HLA to bind inhibitory NKR that signal NK cells to remain in a resting state. NK cell activation not only requires reduced signaling through inhibitory NKR but also activating signals through the interaction of activating NKR with their ligands (5). NK cells can be activated directly when the integration of signals from inhibitory and activating NKR upon binding their ligands on neighboring cells favors activation. They can also be activated in an antibody dependent (AD) manner. Modulation of NK cell activity by antibodies and their ability to interact with dendritic cells to shape adaptive immune responses positions NK cells at the intersection of innate and adaptive immunity (6). NK cells have been implicated in host responses to several viral infections, including to the human immunodeficiency virus (HIV), human cytomegalovirus, hepatitis B virus, hepatitis C virus and influenza virus (7–14). The importance of NK cells in the control of HIV infection is illustrated by the emergence of HIV mutations able to escape NK cell mediated immune pressure (15).

Treatment naïve people living with HIV (PLWH) display a wide range of HIV disease progression rates. Elite Controllers (ECs) are a subset of PLWH at one extreme of this range. They are rare, making up between 0.2% - 0.5% of PLWH. They suppress HIV viral load to below the limit of detection of standard viral load assays [ $<50$  copies/milliliter (ml) of plasma] often maintaining high  $CD4^+$  T-cell counts without treatment (16–21). Viral Controllers (VCs) maintain viral loads of between 50 and either 2000 or 3000 copies/ml of plasma without treatment depending on the cohort studied (16, 18, 19, 22). ECs and VCs are subsets of a larger group known as long term non progressors (LTNPs) that make up approximately 5% of PLWH with the slowest rate of HIV disease progression. Since the term LTNPs was coined before the availability of HIV viral load assays, LTNPs are defined by their ability to maintain elevated  $CD4$  counts for 7 or more years without treatment (16, 22).

ECs and VCs are heterogeneous groups whose classification is based on length of follow up, viral load cut off, presence/absence and frequency of viral load blips and  $CD4$  counts. The specific criteria used to define ECs and VCs differ from one cohort to another (16, 23–25). In this review, we will use the terms “EC” to refer to PLWH who have a viral load of  $<50$  copies/ml plasma and “VC” to refer to PLWH with a viral load of between 50 and 3000 copies/ml plasma with rare viral load blips (16). Terms such as slow progressors, LTNPs, HIV controllers (HIC) and controllers have been used to describe individuals who are not strictly ECs or VCs, as defined by these criteria. In this review, we will use the term “controller” to refer to individuals who are not ECs or VCs. The term “non-controllers” will be used to identify individuals not meeting the criteria for classification as “ECs”, “VCs” or “controllers”. Non-controllers are PLWH who are in the chronic phase of HIV infection and are either untreated, viremic progressors or treated PLWH. There also exist a group of PLWH who started anti-retroviral therapy (ART) early in infection, remained on ART for various durations and who were able to control their viral load after

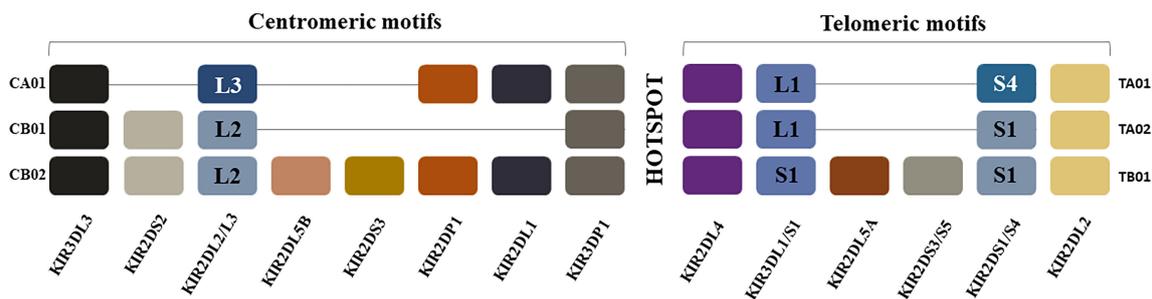
ART was stopped (26). As little information is available on a role for NK cells in these post-treatment controllers, they will not be discussed further in this review.

Several mechanisms have been proposed to underlie HIV control. Among these are infection with defective virus (18, 27, 28) and anti-virus-specific immune responses (29–31). HIV-specific  $CD8^+$  T cell responses in controllers are more polyfunctional and more likely to degranulate and release perforin and granzyme B upon stimulation than  $CD8^+$  T cells from non-controllers (32–34). HIV-specific  $CD4^+$  T cells from controllers are more likely to secrete Interleukin (IL)-2 and proliferate upon stimulation than those from non-controllers (35–37). The HIV proviral landscape in ECs differs from that in successfully treated PLWH. ECs have fewer proviral sequences than PLWH on ART, but the proportion of proviruses that are intact and likely to be replication competent is higher in EC than in treated PLWH (38). In ECs, compared to treated PLWH, HIV provirus is more likely to integrate into genetic regions characterized by deep latency, where reactivation is unlikely (38). Should reactivation occur, HIV from ECs express intact gene products targeted by the potent T cell responses often seen in this population. Some ECs can suppress HIV very early after infection, possibly before the emergence of adaptive immune responses (39, 40). Such early control suggests that innate responses may be involved in early HIV control in some ECs (41). This review will explore the role of NK cell mediated anti-HIV responses in HIV viral control.

## NKR-LIGAND PAIRS - GENETICS, STRUCTURE AND NK CELL EDUCATION

Genome wide association studies (GWAS) found several single nucleotide polymorphisms (SNPs) associated with viral load control that mapped to the major histocompatibility complex (MHC) on human chromosome 6 (42, 43). A SNP within an endogenous retroviral element associated with the gene encoding the protective HLA-B\*57:01 antigen and a second one 35 kilobases upstream of *HLA-C* accounted for nearly 15% of the variation in viral load setpoint in untreated PLWH (42). A second GWAS confirmed these results and identified additional MHC class I alleles associated with protection and risk of HIV disease progression (43). These specific associations with HIV control mapped to amino acids within the MHC class I's peptide binding groove implicating an important role for  $CD8^+$  T cell responses in HIV control (32–34, 43). It should be noted, however, that many HLA allotypes also interact with inhibitory killer immunoglobulin-like receptors (KIRs) expressed on NK cells and other lymphocyte subsets (44, 45).

KIR and HLA are both encoded by highly polymorphic and polygenic genetic regions on chromosomes that segregate independently from each other allowing for a great diversity of receptor ligand combinations (46, 47). KIRs are encoded by up to 15 distinct polymorphic genes within the leukocyte receptor complex on human chromosome 19q13.4 (48) (**Figure 1**). KIRs share structural features, They can have two or three



**FIGURE 1** | Killer Immunoglobulin-like Receptor Haplotypes. *KIR* genes are organized into haplotypes A and B. *KIR* haplotype A is comprised of four framework genes present in most *KIR* haplotypes (*KIR3DL3* at the centromeric end, *KIR3DL2* at the telomeric end and the pseudogene *KIR3DP1* and *KIR2DL4* in the middle) plus genes encoding inhibitory KIRs *KIR2DL1*, *KIR2DL3*, *KIR3DL1*, activating KIR *KIR2DS4* and pseudogene *KIR2DP1*. The more diverse group B haplotypes include the framework genes with various combinations of genes encoding inhibitory KIRs *KIR2DL2*, *KIR2DL5A/B* and activating KIRs *KIR2DS1*, *KIR2DS2*, *KIR2DS3*, *KIR2DS5* and *KIR3DS1*. Most *KIR* region haplotypes are composed of one of 3 centromeric and one of 3 telomeric *KIR* motifs that include combinations of *KIR* genes in linkage disequilibrium with each other, which are usually inherited together. The hotspot in the center between the centromeric and telomeric regions allows for frequent recombination between the two regions. The centromeric region is delimited by the framework genes *KIR3DL3* and *KIR3DP1* while the telomeric region is delimited by framework genes *KIR2DL4* and *KIR3DL2*. *KIR2DP1* and *KIR2DP1* are pseudogenes. CA01 = genes in the centromeric region of *KIR* haplotype A 1; CB01 = genes in the centromeric region of *KIR* haplotype B 1; CB02 = genes in the centromeric region of *KIR* haplotype B 2; TA01 = genes in the telomeric region of *KIR* haplotype A 1; TA02 = genes in the telomeric region of *KIR* haplotype A 2; TB01 genes in the telomeric region of *KIR* haplotype B 1.

extracellular immunoglobulin-like domains (i.e. 2D or 3D in the protein's name) and either long (L) or short (S) cytoplasmic domains, transmitting inhibitory or activating signals, respectively (49). "P" in the *KIR* nomenclature refers to pseudogene. Both inhibitory and activating receptors can contribute to NK cell education. Education through inhibitory receptors reduce the threshold for NK cell responses to missing-self cells while education through activating receptors dampen NK cell activation levels (2, 50).

The *KIR* gene locus is classified into *KIR-A* and *KIR-B* haplotypes, which are distinguished by their activating *KIR* gene content (Figure 1) (46, 51, 52). *KIR* haplotypes share 3 framework genes and 1 pseudogene. *KIR3DL3* and *KIR3DP1* flank the centromeric *KIR* region genes, while *KIR3DL2* and *KIR2DL4* flank the telomeric *KIR* region genes. *KIR2DP1* and *KIR2DL4* are separated from each other by an intergenic region, which is a recombination hotspot. The genes in the *KIR-A* haplotype encode inhibitory receptors, with the exception of *KIR2DS4* receptor, which encodes an activating receptor that is often truncated and not cell surface expressed (53, 54). *KIR-B* haplotypes include the framework genes and varying numbers of activating *KIR* genes. The genes in the telomeric and centromeric regions are in linkage disequilibrium with each other and tend to be inherited together.

The ligands for inhibitory NKR are a subset of HLA antigens (Table 1). *KIR3DL1* is an inhibitory receptor that interacts with the subset of HLA-A and -B antigens carrying the serological Bw4 motif (55, 56). Bw4 antigens have either an isoleucine (\*80I) or a threonine (\*80T) at position 80 of the HLA heavy chain, which influences their cell surface expression density and ligand binding affinity (57–59). Bw4\*80I variants are usually expressed at a higher density than Bw4\*80T antigens (59). The remaining HLA-B antigens have a HLA-Bw6 motif that does not interact with *KIR3DL1* such that NK cells expressing *KIR3DL1* as their

only NKR remain uneducated in *HLA-Bw6* homozygotes (60). The *KIR3DL1/S1* locus encodes inhibitory KIRs expressed at high (*KIR3DL1*-high) and low (*KIR3DL1*-low) cell surface densities and having no cell surface expression (*KIR3DL1*\*004). *KIR3DS1*, also encoded at this locus is an activating receptor (59, 61). In general, *KIR3DL1*-high receptors have a higher affinity for Bw4\*80I than Bw4\*80T allotypes while the affinity of *KIR3DL1*-low receptors for Bw4\*80I and Bw4\*80T antigens is similar (59).

The inhibitory KIRs, *KIR2DL1*, *KIR2DL2* and *KIR2DL3* use HLA-C allotypes as ligands. HLA-C allotypes can be dichotomized into HLA-C1 and HLA-C2 groups based on whether they have an asparagine or lysine at position 80 of their heavy chain. C1 allotypes are ligands for *KIR2DL3* (62). C2 allotypes are ligands for *KIR2DL1* and *KIR2DS1* (63–65). *KIR2DL2* is an intermediate receptor, which also binds HLA-C1 and some HLA-C2 allotypes (64).

Besides KIRs, other inhibitory NKR contribute to NK cell education and tolerance to self. The NK group 2 member A (NKG2A)/CD94 heterodimer are C-type lectin receptors that recognize epitopes from the leader region of HLA-A, -B, -C and -G presented by the MHC class Ib HLA-E antigen (Table 1) (66–68). The leukocyte immunoglobulin-like receptor 1 (LIR-1) also interacts with conserved regions of HLA antigens (69). The signaling lymphocytic activation molecule (SLAM) family can provide NK cells with either activating or inhibitory signals (70).

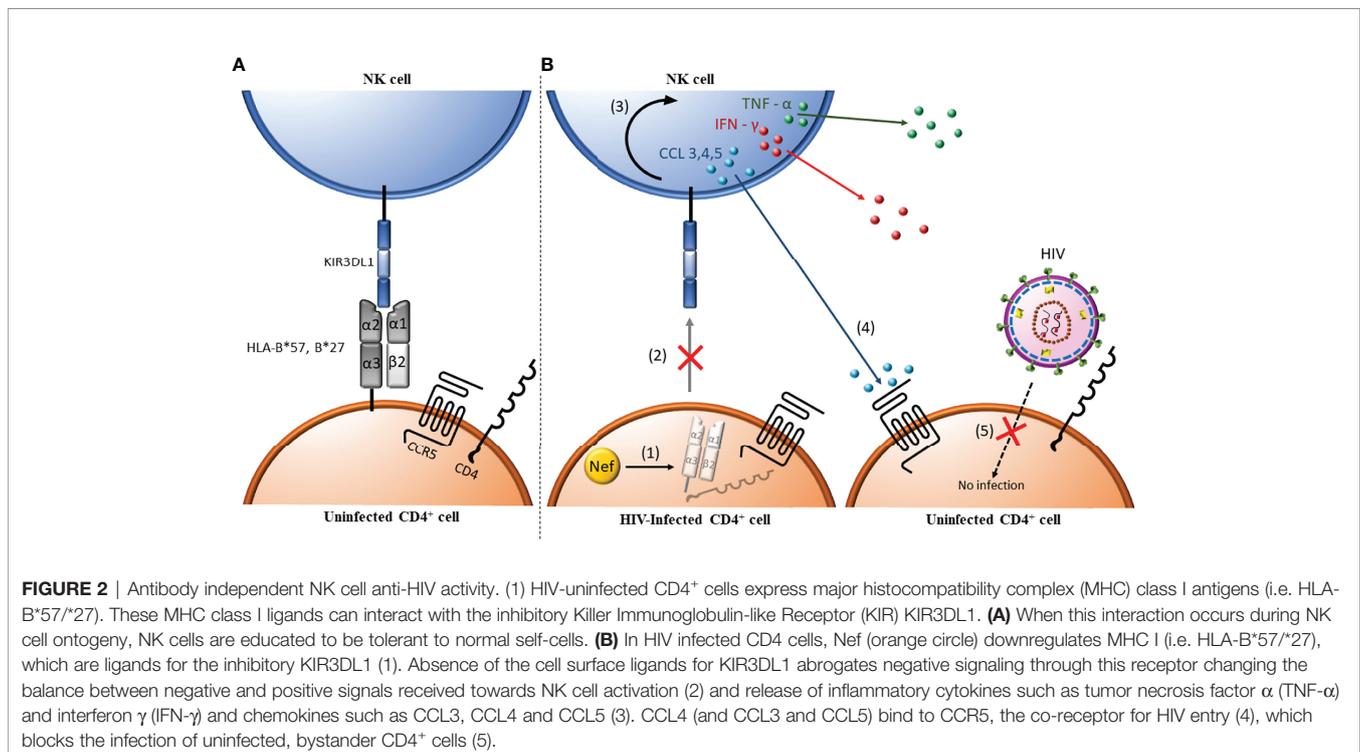
Binding of inhibitory NKR to their HLA ligands transduces intracellular inhibitory signals that generally dominate activating ones to support tolerance to healthy HLA expressing autologous cells (5, 71, 72). NK cell education occurs when inhibitory NKR<sup>+</sup> NK cells interact with healthy self HLA<sup>+</sup> cells during development. Education prevents auto-reactivity to self-cells and primes NK cells for activation when they encounter target cells with reduced levels of HLA ligands as can occur in virus

**TABLE 1** | NK Receptor-Ligand Pairs.

Inhibitory receptor-ligand pairs		Activating receptor-ligand pairs	
Inhibitory Receptor	Ligand	Activating receptor	Ligand
KIR2DL1	HLA-C2 group (Lys <sup>80</sup> )	KIR2DS1	HLA-C2 group (Lys <sup>80</sup> )
KIR2DL2/3	HAL-C1 (Asn <sup>80</sup> )	KIR2DS2	HLA-C1 group, with viral peptide
	HLA-C2 group (Lys <sup>80</sup> ) (variable)		
KIR2DL5A/B	Unknown	KIR2DS3	unknown
KIR2DL3	Unknown	KIR2DS5	HLA-C2 (variable)
KIR3DL1	HLA-A and HLA-B allotypes with Bw4 motifs	KIR3DS1	HLA-F
KIR3DL2	HLA-A*03 and HLA-A*11	KIR2DS4	Subsets of HLA-A*03 and HLA-A*11
NKG2A/CD94	HLA class I signal peptides presented by HLA-E	KIR2DL4	HLA-G
LILRB1	HLA class I	CD16	Immunoglobulin G Fc region
		NKp30	B7 family member B7-H6, HLA-B associated transcript 3 (Bat-3)
		NKp44	heparan sulfate, proteoglycan, proliferating cell nuclear antigen
		NKp46	heparan sulfate, proteoglycan, heparin, vimentin
		NKG2C	HLA-E complexed with the peptides from the leader sequence of HLA-A, -B, -C, and -G and Human cytomegalovirus UL40

infected, transformed and stressed or damaged cells (73) (**Figure 2**). NK cell education levels depend on the number of inhibitory NKR-ligand interactions, inhibitory NKR-ligand density and the avidity of this interaction, which, in turn, correlates with the level of activation mature NK cells achieve when they encounter cells with aberrant HLA expression levels (2, 59, 74). NKRs are expressed stochastically on overlapping NK

cell subsets. Thus, both educated and uneducated NK cells can co-exist as can NK cells with varied levels of education and responses to stimulation, due to allelic *NKR* and *HLA* variation (57, 59, 64). The inhibitory *NKR*<sup>+</sup> NK cell subsets unable to interact with self HLA during development remain uneducated and are hyporesponsive unless activating signals predominate (4, 60, 75, 76).



## KIR/HLA RECEPTOR LIGAND COMBINATIONS IN HIV CONTROL

Epidemiological studies have identified specific *KIR/HLA* combined genotypes that influence the outcome of HIV infection (12, 13). Inhibitory KIR3DL1 and its activating KIR3DS1 counterpart are associated with slower time to the acquired immunodeficiency syndrome (AIDS) (using the 1987 definition for AIDS of the US Centers for Disease Control and Prevention), time to CD4 counts of >200 cells/mm<sup>3</sup>, HIV viral load control and in the case of KIR3DS1 protection against opportunistic infection late in disease, when co-expressed with certain Bw4 allotypes (12, 13, 77). In these studies, Bw6 homozygotes with uneducated KIR3DL1<sup>+</sup> NK cells served as controls for the effect of educated KIR3DL1<sup>+</sup> NK cells on HIV outcomes.

The protective *HLA-B\*57* and *HLA-B\*27* alleles are present in a higher frequency of controllers than non-controllers (43, 78–81). The *KIR3DL1\*h/\*y + B\*57* genotype encodes at least 1 copy of a KIR3DL1-high receptor. This combination conferred the strongest protection against HIV disease progression compared to Bw6 homozygotes (13). This is consistent with *HLA-B\*57*'s protective effect on HIV disease outcome not only being due to its function in restricting CD8<sup>+</sup> T cells responses but also to its role in mediating protective NK cell responses to HIV. While *B\*57* is enriched in ECs, most *B\*57*<sup>+</sup> PLWH fail to control their infection and progress to AIDS at a rate indistinguishable from that seen in PLWH without *B\*57* (16, 35, 78). A nonsynonymous variant of the KIR3DL1 (I47V) with a valine rather than an isoleucine at codon 47 modifies the protection conferred by *B\*57:01* but not *B\*57:03* (82). The rs643347G SNP which encodes the KIR3DL1 47V variant is associated with EC status, lower viral loads and higher CD4 counts (82).

The *KIR3DL1\*l/\*x + B\*27* combined genotype, where *l/\*x* encodes either two copies of KIR3DL1-low receptors or one KIR3DL1-low and a KIR3DL1\*004 null receptor, also confers protection against HIV disease progression, though the protective effect is not as strong as the one associated with the *KIR3DL1\*h/\*y + B\*57* genotype combination (13). Other *KIR/HLA* combinations associated with slower time to AIDS and lower viral load than seen in Bw6 homozygotes include *KIR3DL1\*004 + Bw4*, *KIR3DL1\*h/\*y + Bw4\*80I*, *KIR3DL1\*h/\*y + B\*27*, *KIR3DL1\*l/\*x + B\*57* and *KIR3DS1 + Bw4\*80I* (12, 13).

The involvement of HLA-C in HIV control has been controversial (16, 83). Some have questioned whether any influence attributed to HLA-C on HIV control could be explained by its linkage disequilibrium with protective HLA-B allotypes such as *B\*57:01* (80). On the other hand, there is support for the independent involvement of HLA-C molecules in HIV-1 infection outcomes (84, 85). GWAS studies found that SNP rs9264942 located 35 kilobases upstream of the HLA-C locus was associated with HIV viral load control (42, 43), slower progression to AIDS (86) and HLA-C cell surface expression levels in Caucasians (87). The rs9264942 SNP is marking another SNP, rs67384697 in the 3' untranslated region of *HLA-C*, encoding either guanine or a deleted base pair at codon 263

mapping to the 3' untranslated region of HLA-C that affects the binding of the microRNA miR-148a to this site (88). Weakly expressed HLA-C allotypes possess an intact miR-148a binding site (i.e. a guanine nucleotide at codon 263) while HLA-C antigens expressed at high levels have a deletion at this site, which prevents binding of this microRNA (88). Independent evidence for a causal effect of HLA-C expression levels on HIV control comes from the correlation between the frequency of escape mutations in predicted HLA-C epitopes and viral load (89, 90) and from the strong positive correlation between the frequency of HLA-C peptide-restricted CTL responses and HLA-C expression levels (90). It is not clear whether the immune mechanisms of HIV protection associated with these SNPs involve NK cells.

Malnati et al. compared Caucasian ECs with undetectable viral loads and LTNPs with persistent detectable viremia for genetic markers on chromosome 6 in combination with their KIR genotypes (91). They found that genotypes encoding HLA-C2 groups antigens expressed at high cell surface levels, due to their having *HLA-C2* alleles insensitive to miR-148 regulation, co-carried with telomeric *KIR-B* haplotype genes encoding activating KIR3DL1 and KIR2DS receptors were a hallmark of EC status. In contrast, homozygosity for *HLA-Bw4\*80I* but not homozygosity for the HLA-C linked rs67384697 243/del SNP predicted LTNPs status. Classification as an LTNP was not associated with the presence of telomeric activating KIRs. These findings implicate a role for activating KIR2DS receptors in HIV elite control.

The ligand for the inhibitory NKG2A NKR is HLA-E, stabilized by signal peptides originating from the leader sequence of HLA-A, -B and -C molecules corresponding to amino acids -22 to -14 (66, 67, 92) (**Table 1**). Position 2 of this signal peptide can be either a methionine (-21M) or a threonine (-21T) (93, 94). All HLA-A and HLA-C isotypes are -21M, while HLA-B antigens can be dichotomized into -21M or -21T variants (94). The HLA-B -21 M/T variant distinguishes two types of HLA haplotypes, each having distinct influences on NK cell education. HLA-B -21M variants contribute more effectively to NKG2A mediated education while HLA-B -21T variants preferentially contribute to education through inhibitory KIRs (94). As *HLA-B -21M* and *HLA-Bw6/HLA-C1* are in linkage disequilibrium, the HLA antigens encoded by -21M *HLA-B* haplotypes are usually HLA-Bw6 positive, which does not interact with KIR3DL1 and HLA-C1 antigens are often expressed at low cell surface densities and interact with KIR2DL2/3 with lower avidities than do HLA-C2 with KIR2DL1, consequently affecting the potency of NK cell education and NK cell activation upon missing-self recognition.

HLA-A expression levels vary from one allotype to another (95). A higher density of HLA-A expression is associated with worse prognosis in PLWH (95). HLA-A expression levels distinguish ECs from non-controllers (95). *HLA* haplotypes that carry *HLA-B -21M* and high expression *HLA-A* alleles encode HLA antigens that preferentially provide epitopes that stabilize cell surface HLA-E expression, augmenting its cell surface expression, such as on HIV infected cells; this biases

NK cell education through inhibitory NKG2A (94). Once NKG2A<sup>+</sup> NK cells, educated in this manner, encounter HIV infected cells expressing high levels of HLA-E, inhibition of NK cell effector function ensues, which negatively impacts HIV control (13, 95).

In adult HIV infection, the most important immunogenetic factors contributing to time to AIDS and HIV viral load are the protective and disease-susceptible HLA-B allotypes (37, 42, 96, 97). Several *KIR3DL1* + *Bw4* combinations also contribute to more favorable HIV outcomes in adults (13, 98). In contrast, in untreated pediatric HIV infection, the HLA-B antigens that are protective in adults do not significantly contribute to HIV outcomes (98). A study performed on >300 children from sub-Saharan Africa classified children as progressors, slow progressors who were viral non-controllers and slow progressors who were viral controllers. This classification was based on how old the children were when they reached pre-determined CD4 count criteria to start ART if this occurred after five years of age and whether their plasma viral load was <10,000 versus >10,000 RNA copies/ml plasma (98). Carriage of *Bw4* and low expression *HLA-A* alleles were strongly associated with HIV control and slow HIV disease progression in this pediatric cohort (98, 99). As carriage of *KIR3DS1* is rare in many African populations, *Bw4* is likely co-expressed with *KIR3DL1*, which educates these NK cells. The viral controller children, compared to the other pediatric groups, were likely to be controlling HIV through NK cell responses using a KIR based education program.

## LINKS BETWEEN *KIR/HLA* GENOTYPES AND NK CELL FUNCTIONAL POTENTIAL

When HLA-null cells such as 721.221 and K562 cells stimulate NK cells, negative signaling through inhibitory KIRs is abrogated while signals through activating receptors binding their ligands on HLA-null cells persist. In this scenario, NK cells are activated in accordance with the potency with which they were educated, revealing their functional potential. HLA-null cell stimulation of NK cells from carriers of the *KIR3DL1-high* + *HLA-B\*57* genotype combination activates a higher frequency of NK cells to secrete IFN- $\gamma$ , the chemokine CC motif ligand 4 (CCL4) and externalize the degranulation marker CD107a than NK cells from carriers of other *KIR3DL1/HLA* combinations or from *Bw6* homozygotes (100). This is the case for NK cells from both HIV uninfected persons (100) and from PLWH (101). It should be noted, however, that HIV infection has a negative impact on NK cell functionality, though NK cells from controllers have higher functional potential than those from non-controllers (102–106).

NK cells from individuals carrying the *KIR3DL1* + *HLA-B\*27* and *KIR3DL1\*004* + *HLA-Bw4* genotype combinations had higher functional potential when stimulated with HLA-null cells than those from *Bw6* homozygotes (107, 108). The latter finding suggests that the *KIR3DL1*-null allotype *KIR3DL1\*004*, while not expressed on the surface of NK cells, does support NK cell education (109, 110). Korner et al. showed a similar pattern

of responses to HLA-null cells for *KIR2DL*<sup>+</sup> NK cells (111). A higher frequency of NK cells educated though *KIR2DL1* and *KIR2DL3* than their uneducated counterparts responded HLA-null 721.221 cell stimulation by secreting tumor necrosis factor alpha (TNF- $\alpha$ ) and externalizing CD107a (111).

## *KIR/HLA* GENOTYPES INFLUENCE RESPONSES TO AUTOLOGOUS HIV INFECTED CD4 T CELLS

The HIV negative regulatory factor (Nef) and the viral protein U (Vpu) are HIV encoded accessory protein with several functions, including the ability to down-modulate HLA-A, -B and -C from the surface of HIV infected cells (112–116). Most HIV isolates can reduce the levels of HLA-A and -B expression while HIV isolates differ in their ability to downmodulate HLA-C (111, 112, 114, 117). The lower HLA antigen expression levels on infected CD4 cells reduces signaling through inhibitory NKRrs on autologous educated NK cells, which leads to their activation (112, 114). NK cells, activated by missing-self recognition of autologous HIV infected cells, control HIV replication, at least in part, by secreting soluble CCL3, CCL4, and CCL5, which binds the C-C chemokine receptor 5 (CCR5), the co-receptor for HIV entry, thus blocking the infection of new HIV susceptible CD4 target cells (118, 119).

Inhibitory *KIR*<sup>+</sup> NK cells educated through interactions with their HLA ligands on neighboring cells have higher responses to autologous HIV-infected CD4 cells than their uneducated counterparts. This can be observed by gating on NK cells expressing a single inhibitory *KIR* by flow cytometry. The frequency of single positive *KIR3DL1* NK cells secreting IFN- $\gamma$ , CCL4 and externalizing CD107a, all of which have anti-HIV activity is higher in educated, than uneducated, *KIR3DL1*<sup>+</sup> NK cells responding to autologous HIV-infected CD4 cells stimulation (117). Expression levels of both the *KIR3DL1* receptor and its HLA-B ligand and their binding affinity influence single positive *KIR3DL1*<sup>+</sup> NK cell education potency and responsiveness to autologous HIV-infected CD4 cells (59, 117). For example, NK cells from *KIR3DL1-high* + *HLA-B\*57* carriers have a superior ability to inhibit HIV replication in autologous infected CD4 cells compared to carriers of other *KIR/HLA* combinations and *Bw6* homozygotes (118).

Carriage of the *KIR3DS1* + *HLA-Bw4\*80I* allele combination, associated with slower time to AIDS produces NK cells with a superior ability to suppress HIV replication in autologous HIV-infected CD4 cells compared to carriers of either the receptor or ligand alone or neither (12, 77, 120). Direct evidence for an interaction between *KIR3DS1* and most HLA-*Bw4\*80I* antigens is lacking with the exception of one report showing that *KIR3DS1* interacted with HLA-B\*57, if certain HIV peptides were present (121–124). *KIR3DS1* binds the open conformation of the non-classical MHC class Ib antigen, HLA-F (121). NK cells stimulated by the HLA-F expressing HLA-null cell line, 721.221 and autologous HIV-infected CD4 cells, stimulated a higher frequency of NK cells to produce IFN- $\gamma$ , CCL4 and CD107a

than control non-HLA-F expressing stimuli (121, 125, 126). Blocking this interaction with either antibodies to HLA-F or KIR3DS1-Fc chimeric proteins significantly reduced KIR3DS1<sup>+</sup> NK cell activation. Further investigations are needed to understand the anti-viral activity of KIR3DS1<sup>+</sup> NK cells from individuals who are HLA-B\*80I<sup>+</sup> given that interactions between this receptor and ligand pair have been challenging to demonstrate experimentally.

NK cell education through HLA-C ligand/inhibitory KIR2DL receptor interactions, also contribute to responsiveness to autologous HIV infected cells. As was the case for single positive KIR3DL1 NK cells, a higher frequency of educated than uneducated single positive KIR2DL1, KIR2DL2 and KIR2DL3 NK cells responded to autologous HIV-infected CD4 cells by producing IFN- $\gamma$ , CCL4 and CD107a with the strongest effect seen for CCL4 secretion (117). Similar results were observed for KIR2DL1<sup>+</sup> and KIR2DL3<sup>+</sup> NK cells responding to co-cultured autologous HIV infected cells, using inhibition of viral replication as a read out. Educated NK KIR2DL1<sup>+</sup> and KIR2DL3<sup>+</sup> cells also had a superior ability, compared to their uneducated counterparts, to reduce viral load levels in these co-cultures (111).

In summary, NK cell interactions with autologous HIV-infected CD4 cells stimulate a higher frequency of educated than uneducated NK cells to elicit functions that control HIV. The frequency of NK cells secreting CCL4 appears to be particularly important in discriminating inhibitory KIR/HLA combinations with variations in education potency (117). Larger study populations will be required to determine whether individual protective HLA alleles identified in epidemiological studies and by GWAS educate NK cells for anti-HIV activity, as was observed for HLA-null cell stimulation of NK cell (13, 42, 43, 100, 101, 117).

## ASSOCIATION BETWEEN NK CELL ACTIVITY AND THE HIV RESERVOIR SIZE

The KIR/HLA combinations discussed above educate NK cells, which then respond to autologous HIV infected cells with reduced cell surface HLA levels by stimulating functions that control HIV. HIV control is assessed by measuring viral load in supernatant, the frequency of activated NK cells producing anti-HIV functions and the frequency of HIV infected cells in NK cell/HIV infected cell co-cultures. PLWH have proviral DNA integrated into long-lived HIV reservoirs, which are an obstacle to viral clearance and HIV cure (127). ECs who exhibit the features of a functional HIV cure, are distinguished from successfully treated PLWH by having smaller HIV viral reservoirs (128). The size of the HIV proviral reservoir is thought to reflect immune mechanisms that contain HIV replication. What these mechanisms are is an active area of investigation. There is some evidence that NK cell subsets play a role in shaping the size of the integrated HIV DNA reservoir.

Using mass cytometry to measure the expression of twenty-four cell surface markers on peripheral NK cells, two machine

learning approaches were used to identify NK cell subpopulations that distinguished ECs from untreated, viremic, non-controllers and that correlated with the size of integrated HIV DNA reservoir. The frequency of the CD11b<sup>+</sup>, CD57<sup>+</sup>, CD161<sup>+</sup>, Siglec-7<sup>+</sup> subset of CD56<sup>dim</sup> CD16<sup>+</sup> NK cells was significantly more abundant in ECs and HIV uninfected controls than in untreated, viremic non-controllers and correlated with cell associated proviral HIV DNA levels (129). These markers identify highly active, partially mature, cytotoxic NK cells with proliferative capacity and responsiveness to cytokines such as IL-12/IL-18. This NK cell sub-population had an enhanced ability to respond to IL-12/IL-18 stimulation by secreting IFN- $\gamma$  and to HLA-null cell stimulation by externalization of CD107a compared to CD11b<sup>+</sup>, CD161<sup>+</sup> or Siglec-7<sup>+</sup> CD56<sup>dim</sup> CD16<sup>+</sup> NK cells (129). The activity of this subpopulation may be contributing to HIV control in untreated ECs.

Marras et al. investigated NK cell functional features that were inversely correlated with total and integrated HIV DNA copy number in circulating CD4 T cells (128). The higher the frequency of NK cells induced to secrete IFN- $\gamma$  following IL-2 and IL-15 stimulation and the higher the induction of activating NKp30/NKp46 natural cytotoxicity receptors over baseline by IL-2, the smaller the HIV reservoir size. The fold increase over background in induced NKp30/NKp46 expression positively correlated with the cytotoxic potential of these NK cells. NK cells from controllers and treated non-controller PLWH differed in the transcriptional program of their IL-2 induced NK cells. The functional pathways induced in NK cells from controllers upon IL-2 stimulation involved IFN- $\gamma$ , IL-2/IL-12 and TNF- $\alpha$  signaling pathways, the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) pathway, lysosome signaling and cytolysis. Activated NK cells from controllers, but not from treated PLWH, dramatically suppressed viral replication and reduced levels of integrated HIV DNA in NK cell/infected CD4 T cell co-cultures (128). The control of HIV DNA copies observed predominantly in ECs supports a role for *in vivo* NK cell function in this control, even when these NK cells fail to control viral load and HIV replication.

## ANTIBODY DEPENDENT NK CELL ACTIVITY IN HIV CONTROL

Most CD56<sup>dim</sup> NK cells express the activating immunoglobulin fragment crystallizable (Fc) gamma III a receptor, Fc $\gamma$ IIIa or CD16, that binds the Fc portion of immunoglobulin G (IgG), particularly to IgG1 and IgG3 subtypes (130). The antibody's antigen binding site interacts specifically with antigens, such as those on the surface of target cells while the antibody Fc region binds and cross-links CD16 on NK cells. Antibodies thus form a bridge between NK cells and target cells, which activates NK cells to secrete cytokines, chemokines and to externalize CD107a, which are measured by AD NK cell activation (ADNKA). ADCC measures the cytolysis arising from the activation of NK cells through this interaction. Although other cell types such as

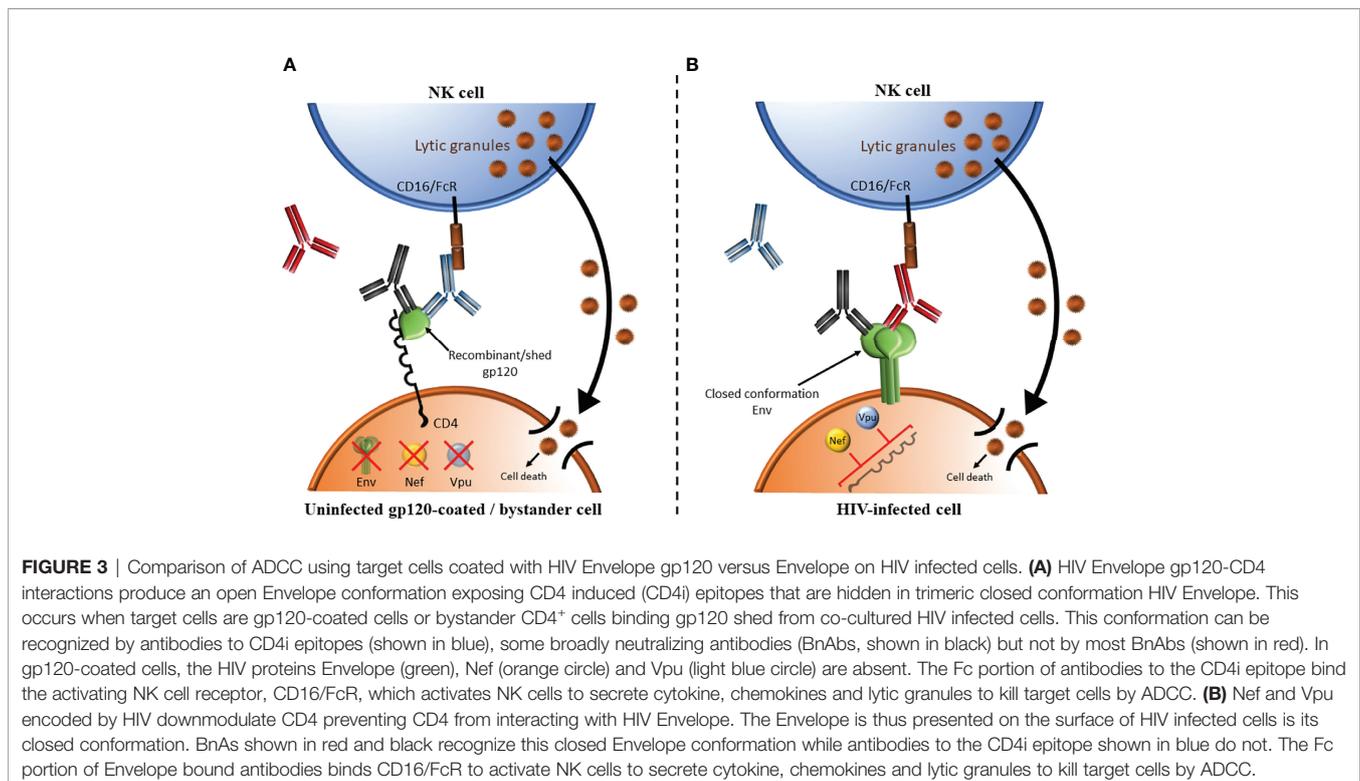
macrophages, resident monocytes, neutrophils and eosinophils can also mediate ADCC, NK cells are considered the most important cell type mediating this activity.

Antibody binding to CD16 can be modified by glycosylation patterns with afucosylated antibodies having the strongest affinity for CD16 (131, 132). There can be regulation of ADCC activity at the effector cell level since there are two major allelic variants of CD16, which are distinguished by a SNP that affects ADCC activity (133, 134). A valine at amino acid 158 of CD16 confers enhanced affinity for IgG1 and IgG3 compared to a phenylalanine at this position (130). Individuals expressing the V158 variant have enhanced ADCC activity (135).

### Methodological Considerations – Antibodies, Target Cells and ADCC/ADNKA Assays

AD functional assays targeting HIV infected cells depend on the presence of antibodies specific for HIV Envelope, the only gene product expressed on the surface of infected cells and virions (136). Anti-HIV Envelope-specific antibodies are present in all PLWH as well as in recipients of vaccines that include Envelope gp120 antigens in the vaccine regimen (137–141). Most assays measuring the ADCC competence of antibodies in PLWH use either CD4<sup>+</sup> CEM.NKR.CCR5 (CEM) cells coated with monomeric recombinant HIV Envelope gp120 (142–151) or HIV-infected cells (152–155) as target cells. In both cases, CD4-induced (CD4i) epitopes that are normally hidden within the native trimeric Envelope expressed on genuinely HIV infected cells are exposed while the CD4 binding site (CD4bs) epitopes are occluded by binding CD4 expressed on coated cells

CEM cells or on uninfected CD4 bystander cells present in the same culture as infected CD4 cells (156). The Envelope's CD4bs epitopes are highly conserved and antibodies to this epitope are among the most potent broadly neutralizing antibodies (Figure 3). HIV Envelope gp120-CD4 interactions produce an open Envelope conformation exposing CD4i epitopes that are hidden in trimeric closed conformation HIV Envelope (140, 157). CD4i epitopes are exposed on gp120-coated target cells and bystander CD4<sup>+</sup> cells that have bound gp120 shed from co-cultured HIV infected cells (156, 158). The open Envelope conformation is recognized by antibodies to CD4i epitopes such as A32, some broadly neutralizing antibodies but not by most broadly neutralizing antibodies (154–156). Since gp120-coated CEM cells are not infected, the HIV Envelope, Nef and Vpu are absent. The Fc portion of antibodies to the CD4i epitope binds the activating NK cell receptor, CD16, which activates NK cells to secrete cytokine, chemokines and externalize lytic granules to kill target cells by ADCC. In infected cells, HIV Nef and Vpu downmodulate CD4 preventing it from interacting with HIV Envelope (159). The Envelope is thus presented on the surface of HIV infected cells in its closed conformation (155, 156). Most broadly neutralizing antibodies recognize the closed Envelope conformation while antibodies to the CD4i epitope do not (155, 156). The Fc portion of Envelope bound antibodies binds CD16 to activate NK cells to secrete cytokine, chemokines and lytic granules to kill target cells by ADCC. We generated HIV-infected CEM cells that were sorted to exclude uninfected bystander cells. The advantage of using these sorted, infected CEM (siCEM) cells as target cells is that they express Envelope in a closed conformation that resembles what is present on



genuinely HIV-infected cells in which the CD4i epitope is hidden and CD4bs epitopes are exposed (155) (**Figure 3**). Head-to-head comparisons of ADCC assay results using gp120-coated CEM and siCEM cells correlate with each other. However, the abundance of antibodies specific for gp120 on coated CEM cells is 7-fold or more, higher than that of antibodies specific for the closed Envelope conformation on siCEM cells (155, 160).

A plethora of assays have been developed to measure ADCC activity. The earliest assays used was <sup>51</sup>Chromium release from gp120-coated target as a read out for ADCC activity. Some of the disadvantages of the <sup>51</sup>Chromium release assay include the use of radioisotopes and challenges related to the quantification of target cell death. Using <sup>51</sup>Chromium release assays, plasma from controllers and untreated, viremic non-controllers bound similarly to a panel of HIV Envelope antigens. Higher ADCC activity was detected in plasma from controllers than non-controllers using gp120-coated CEM cells (146). The flow cytometry-based rapid and fluorometric ADCC (RFADCC) assay has been widely used to measure ADCC activity (161). This assay measures the loss of carboxyfluorescein succinimidyl ester dye from gp120-coated CEM target cells also stained with the lipophilic membrane dye, PKH-26 (161). The RFADCC assay was eventually shown to measure monocyte mediated AD cellular trogocytosis (ADCT) rather than NK cell mediated ADCC activity as it detected the uptake of PKH-26-stained membranes from the target cells by monocyte effector cells (162). The high throughput, flow cytometry-based GranToxiLux assay measures the frequency of target cells that have taken up granzyme B released from activated NK cells (163). ADCC activity can also be evaluated by measuring the disappearance of live HIV-infected p24<sup>+</sup> target cells by flow cytometry, or decreased luciferase activity when target cells are infected with luciferase-based HIV constructs, or more directly, by measuring apoptosis induction in target cells (139, 140, 154, 155, 164–167). The ADNKA assay measures NK cell activation for secretion of IFN- $\gamma$  and externalization of CD107a; in some assays, secretion of TNF- $\alpha$  and/or CCL4 are also measured (105, 168).

## ADCC AND ADNKA ACTIVITY IN HIV CONTROL

Madhavi et al. compared plasma from 22 controllers and 44 untreated, viremic non-controllers using several ADCC and ADNKA assays (147). Controllers, compared to untreated, viremic non-controllers, had higher levels of gp120-specific antibodies capable of 1) binding CD16, 2) inducing IFN- $\gamma$  secretion and/or CD107a externalization from NK cells in an ADNKA assay and 3) generating granzyme B positive Envelope gp140-coated CEM cells using the GranToxiLux assay (147). The higher level of ADCC activity in plasma from controllers than in untreated, viremic non-controllers measured using the GranToxiLux assay was confirmed by others (163, 169).

In contrast, Ackerman et al. compared ECs VCs, untreated, viremic non-controllers and treated non-controllers in chronic

phase infection for antibodies binding to gp120-coated beads, for activity in the RFADCC assay and for ADNKA activity using gp120-coated CEM cells opsonized with IgG isolated from study subject plasma to stimulate NK cells (142). The titer of plasma anti-gp120 IgG was lower in ECs than that in VCs and untreated, viremic non-controllers. No between-group differences were observed in the RFADCC and ADNKA assays, with the exception that ECs, compared to VCs, had lower activity in the RFADCC assay and a lower frequency of NK cells secreting CCL4 (142). To our knowledge, these functional results were not normalized to the concentration of gp120-specific antibodies present in each of the samples tested.

Using a different study population meeting the same classification criteria, Kant et al. evaluated the concentration of anti-gp120- (using gp120 coated CEM target cells) and anti-Envelope- specific antibodies (using siCEM target cells) in plasma from PLWH. ADCC activity was evaluated using an assay that measured target cell apoptosis, which correlates with target cell cytolysis (138, 155, 170). The concentration of antibodies in plasma from ECs, VCs and untreated, viremic non-controllers that bound gp120-coated CEM cells and Envelope on siCEM cells was similar and higher than the concentration of these antibodies in treated non-controllers. This was also the case for ADCC activity (155). When ADCC assay results were normalized to the concentration of anti-gp120- and anti-Envelope-specific antibodies in each plasma sample, all between-group differences fell below the level of significance. This finding suggested that between-group differences in ADCC competent antibodies were driven by differences in the concentration of the antibodies in plasma able to bind the closed conformation of HIV Envelope on siCEM cells. Thus, the four subject groups did not differ from each other in their per-anti-Envelope-specific antibody ADCC potency (138). Of particular interest, ECs and VCs with an HIV reservoir size that was below the limit of detection had significantly higher normalized ADCC activity measurements than those with a detectable HIV reservoir size, implicating a role for ADCC activity in HIV reservoir size control (138). Others have reported that production of IFN- $\gamma$  by NK cells was associated with lower viral load (171–174) and lower HIV reservoir size (128).

More recent studies have expanded the AD functions tested to include AD cellular phagocytosis (ADCP), AD neutrophil phagocytosis (ADNP), AD complement deposition (ADCD), and ADCT in addition to ADNKA in one study (142) and ADCP, ADCT and ADCD in another (138). Comparisons of these activities in ECs, VCs, untreated, viremic non-controllers and treated non-controllers found minor between-group differences in ADCD and CCL4 secretion (142). As for ADCC activity, plasma from ECs, VCs and untreated, viremic non-controllers generated higher levels of ADCP, ADCT and ADCD activity than plasma from treated non-controllers (138). Between-group differences were lost upon normalizing these values to the concentration of anti-gp120-specific antibodies for ADCP, and anti-Envelope-specific antibodies for ADCT and ADCD. One clear difference in the patterns of anti-gp120-

and anti-Envelope-specific antibodies and in AD functions detected in ECs, VCs, untreated, viremic non-controllers and treated non-controllers was that they were highly correlated with each other in ECs but not in the other groups (138, 142).

A systems serology approach to assess antibody features associated with HIV control included assessments of the antibody specificity to a panel of HIV protein variants, the ability of these antibodies to recruit innate immune cells by determining their titer, Ig class and subclass, antibody Fc glycosylation patterns, antibody binding to Fc gamma receptors, lectin binding properties and AD functionality (175). Anti-HIV-specific antibodies with bisected glycoforms were enriched in ECs compared to untreated, viremic progressors. Gp120-specific antibodies with these glycosylation patterns have a greater capacity to bind CD16 and mediate ADCC. Antibodies specific for HIV p24 were enriched in ECs compared with treated non-controllers as were Fc gamma receptor ligating antibodies to internal HIV gene products other than HIV capsid (175). These observations confirmed previous findings but have the potential to identify novel features of humoral immunity that differentiate ECs from non-controllers, whether on-ART or not. The interest of applying systems serology approaches to compare controllers to non-controllers is its potential to generate new information on what antibody features define functionally potent immunity and to contribute to the identification of mechanisms that correlate with potent AD effector function.

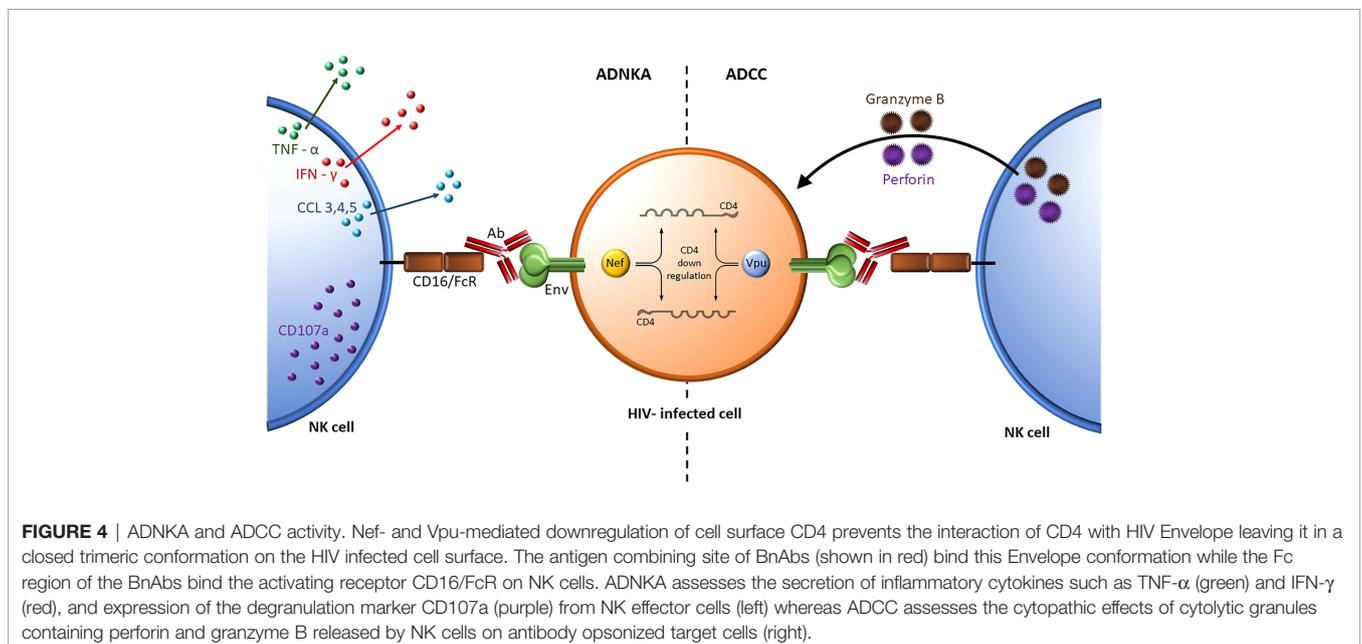
## ROLE OF NK CELL EDUCATION IN ADCC AND ADNKA ACTIVITY

Although many reports refer to ADNKA and ADCC activity interchangeably, the former measures NK effector cell

activation while the later measures the consequences of NK cell activation at the target cell level (**Figure 4**). The role NK cell education in ADCC and ADNKA activity was investigated. ADCC activity was measured using a GranToxiLux assay that assessed the frequency of granzyme B positive gp120-coated CEM target cells opsonized with the same source of antibodies recognizing HIV gp120. The effector cells were NK cells negatively sorted for a single inhibitory KIR that originated from subjects expressing, or not, HLA antigens able to educate NK cells that expressed the inhibitory KIR being sorted for. No differences were observed between educated and uneducated NK cells isolated for a single inhibitory KIR. This was the case for KIR3DL1, KIR2DL1 and KIR2DL2. This finding suggested that education does not play an important role in ADCC activity (176, 177). The reason for this may be due to signaling through the CD16 activating receptor overwhelming any effect on NK cell responsiveness resulting from NK cell education. ADNKA activity was measured as the frequency of NK cells positive for IFN- $\gamma$ , CCL4 and/or CD107a following stimulation by opsonized gp120-coated CEM cells. Unlike ADCC activity, a consistently higher frequency of educated, than uneducated, NK cells responded to this stimulus (176). Together these results underline important differences in these two NK cell functions, which are often referred to interchangeably in the literature (178).

## ADAPTIVE NK CELLS IN HIV VIRAL CONTROL

Human cytomegalovirus (HCMV) infection expands a population of NK cells with adaptive-like features (8). These



**FIGURE 4** | ADNKA and ADCC activity. Nef- and Vpu-mediated downregulation of cell surface CD4 prevents the interaction of CD4 with HIV Envelope leaving it in a closed trimeric conformation on the HIV infected cell surface. The antigen combining site of BnAbs (shown in red) bind this Envelope conformation while the Fc region of the BnAbs bind the activating receptor CD16/FcR on NK cells. ADNKA assesses the secretion of inflammatory cytokines such as TNF- $\alpha$  (green) and IFN- $\gamma$  (red), and expression of the degranulation marker CD107a (purple) from NK effector cells (left) whereas ADCC assesses the cytopathic effects of cytotoxic granules containing perforin and granzyme B released by NK cells on antibody opsonized target cells (right).

adaptive NK (adapNK) cells express the activating receptor, NKG2C, which belongs to the C-type lectin family (179). The NKG2C activating receptor, like its inhibitory counterpart, NKG2A, is expressed as a heterodimer with CD94 (180). The ligand for NKG2C and NKG2A is HLA-E, a nonclassical MHC class Ib antigen cell surface stabilized by peptides derived from leader sequence of classical MHC class I antigens, the nonclassical MHC Ib HLA-G antigen or epitopes derived from the HCMV encoded viral protein UL40 (**Table 1**) (66, 68, 181). The interaction of NKG2C with its ligand transmits signals that activate cells expressing this receptor (66, 182). AdapNK cells undergo DNA methylation-dependent epigenetic modifications, which distinguish them from conventional NK cells and influence their functionality (183, 184).

Some individuals do not express cell surface NKG2C due to a homozygous deletion of approximately 16 kb that includes the *nkg2c* gene encoding NKG2C (185, 186). In several Caucasian populations, a Japanese and a Tanzanian cohort, the frequency of the NKG2C deletion allele is close to 20% with a homozygous deletion genotype frequency of approximately 4% (186–189). Several studies have questioned whether NKG2C<sup>+</sup> cells play a role in HIV viral control in PLWH.

Treatment-naïve viral load set point is associated with the rate of HIV disease progression, as measured by time to CD4 counts of <200/mm<sup>3</sup>, AIDS, and death (190, 191). Thomas et al. found a positive correlation between HIV viral load and the proportion of NKG2C<sup>+</sup> NK cells enumerated at the same time viral load was assessed in a group of 6 NKG2C<sup>+/+</sup> PLWH (192). HCMV status was not assessed in these individuals, which would influence the frequency of NKG2C<sup>+</sup> NK cells. However, most PLWH are HCMV co-infected (193). Ma et al. found a negative correlation between the percentage of NKG2C<sup>+</sup> NK cells and concurrent viral load in 22 treatment-naïve PLWH infected for at least 120 days, which corresponded to the viral load set point (194). Gondois-Rey et al. also found a negative correlation between the percentage of NKG2C<sup>+</sup> NK cells and concurrent viral load in 18 treatment-naïve PLWH tested at time points in acute/early HIV infection (172). Alsulami et al. assessed the pre-treatment viral load set point in 160 NKG2C<sup>+/+</sup>, 83 NKG2C<sup>+/-</sup>, and 6 NKG2C<sup>-/-</sup> PLWH. They found that HIV viral load set points were similar in carriers of the three NKG2C genotypes (137). They also correlated plasma viral load in 43 HCMV seropositive PLWH with the frequency of NKG2C<sup>+</sup> CD56<sup>dim</sup> NK cells and the intensity of NKG2C expression on these cells. Neither the percentage of NKG2C<sup>+</sup> CD56<sup>dim</sup> NK cells nor the intensity of NKG2C expression on these cells was significantly correlated with viral load set point when all observations were considered together or when results were stratified according to NKG2C genotype. The later results suggest no association between the frequency of NKG2C<sup>+</sup> NK cells or the intensity of NKG2C expression on NK cells with viral load control. However, as there are inconsistencies in this finding these results need to be confirmed.

## A ROLE FOR NK CELLS IN VACCINE RESPONSES ABLE TO CONTROL HIV

The only HIV vaccine trial to show significant though modest efficacy against HIV infection was the RV144 HIV vaccine trial (195). Correlates of protection analyses revealed that vaccine induced antibodies able to bind HIV Envelope structures and support NK cell mediated ADCC activity were associated with protection from HIV infection (196). However, a similar vaccine strategy used in a South African population, which induced similar immune responses failed to show efficacy in preventing HIV infection (197). This highlights the importance of investigating strategies that can improve the efficacy of vaccine induced immune responses. ECs spontaneously control HIV without treatment. ECs, compared to non-controllers, are more likely to have polyfunctional HIV-specific T cell responses and highly correlated polyfunctional anti-Envelope antibody dependent activity (32, 142, 170). The RV144 trial provides an example of a role for NK cells in protection from HIV infection while immune responses distinguishing ECs from non-controller implicate a role for NK cells in HIV control.

Early in an immune response, NK cells secrete cytokines and chemokines such as IFN- $\gamma$ , TNF- $\alpha$ , CCL3, CCL4 and CCL5 and granulocyte-macrophage colony-stimulating factor (198). These secreted factors contribute to recruiting and activating antigen presenting cells such as dendritic cells, which in turn induce adaptive immunity (199). IFN- $\gamma$  derived from NK cells can promote type 1 T helper cell differentiation and stimulate isotype class switching in B cells (200, 201). Designing vaccines able to harness the activity of NK cells may be able to improve the induction of adaptive immune responses able to exert HIV control (202), generate innate immune memory (203), and collaborate with other vaccine induced immune responses to increase the prophylactic and therapeutic potential of HIV vaccines (204). The incorporation of novel adjuvants into vaccine formulations may induce NK cell activities that increase adaptive immune responses (205–208).

Most PLWH are also HCMV co-infected (193). CMV infection drives the expansion of adaptive-like NK cells expressing NKG2C and/or that are negative for the FcR $\gamma$  signaling molecule. These adaptive-like NK cells have epigenetic changes that confer AD effector functions such as IFN- $\gamma$  secretion and ADCC activity that is superior to that seen in conventional NK cells (8, 183, 184, 209, 210). Vaccines designed to induce adaptive-like NK cells together with HIV-specific antibodies would be an interesting strategy to consider for controlling several pathogens including HIV and HCMV.

However, NK cells can also negatively regulate immune responses through their cytolytic activity. NK cell mediated cytotoxicity can reduce vaccine antigen persistence, eradicate responding T cells, suppress the establishment of adaptive memory and block the evolution of antibody responses (199, 211, 212). The dichotomous activity of NK cells adds complexity to targeting these cells through vaccination. Future investigations will need to consider this issue when designing vaccines targeting NK cells.

## CONCLUDING REMARKS AND FUTURE DIRECTIONS

Although HIV-specific CD8 T cell responses play a predominant role in HIV control, particularly in adults, there is accumulating and compelling evidence from epidemiological, genetic and functional studies that educated NK cells also contribute to HIV control. In fact, in children, NK cells educated through inhibitory KIR play a more important role than CD8 responses restricted by protective HLA allotype. Certain NK cell subsets are also associated with EC status through less well-defined mechanisms.

A survey of the literature on AD activities in controllers (ECs, VCs) compared to untreated, viremic non-controllers and in some cases treated non-controllers has revealed few consistent patterns of AD functions associated with control other than ECs having more coordinated AD functions than other groups of PLWH. The discrepancies in whether and how AD activities differ in these groups of PLWH may be due to the assays used, what they measure and how results are analyzed. Quantification of antibodies specific for gp120 on coated CEM cells or specific for Envelope on siCEM cells and using these values to normalize AD function results should be applied. The reason for this is that the titer of antibodies specific for monomeric gp120 and trimeric Envelope vary from person to person. ECs, VCs, and untreated, viremic non-controllers have higher titers of these antibodies than do treated non-controllers. The observation that ECs and VCs with undetectable HIV DNA reservoir sizes in circulating CD4<sup>+</sup> T cells have higher levels of ADCC activity than in ECs and VCs with measurable HIV reservoir sizes supports a role for ADCC in HIV control at the HIV reservoir level.

As innate immune cells, NK cells are primed to participate in anti-viral responses and exert antibody independent anti-viral activity at the earliest times post infection and certainly earlier than anti-viral T cell activity can be induced. NK cells can only exercise anti-viral functions through ADCC and ADNKA once antibodies specific for HIV gp120/Envelope appear. Future research should more thoroughly evaluate whether antibody independent NK cell responses are more likely to contribute to the functional HIV cure phenotype exhibited by ECs than AD activities. Future investigations of the anti-HIV activity of NK cells should include the evaluation of HIV reservoir size in addition to viral load in plasma and co-cultures, viral replication, and the frequency of circulating HIV infected cells. It is important at this time to remain open to the possibility that multiple NK cell dependent mechanisms may be involved in HIV control in ECs.

An interesting feature of ECs, compared to treated PLWH, is their ability to maintain high levels of potent anti-gp120- and anti-Envelope-specific antibodies with ADNKA/ADCC functionality in the absence of detectable viremia. Initiation of ART in untreated, viremic non-controllers leads to a decline in HIV-specific immune responses as seen in treated non-controllers (170). This may in part be due to the higher levels of gp-120/Envelope-specific memory B cells maintained in ECs compared to treated PLWH (213–215). This would be an important area for further investigation.

There are many challenges to these studies. Controllers, particularly ECs, are a rare subset of PLWH, impeding recruitment of large EC populations. Furthermore, it has become clear that ECs are a heterogeneous population further hampering the recruitment of homogeneous populations for investigation. There are discrepancies from one cohort to another in how controllers are defined in terms of their viral load cut offs, CD4 counts, length of follow up, among other parameters. The wide range of assays used to measure AD functions, the target cells used to measure ADCC activity and the use of ADCC and ADNKA interchangeably likely also contribute to discrepant results between studies. Overall, the assays used to assess NK cell mediated AD functionality require further standardization in order to better elucidate the role of NK cells in mediating HIV control.

## AUTHOR CONTRIBUTIONS

NB, SK and FD performed the literature review. NB prepared the first draft of the manuscript. SK, ZK, CT, and FD were involved in critical discussions of the content, writing, and editing of the manuscript. SK, FD and NB were involved in the preparation of figures. All authors contributed to the manuscript and approved the submitted version.

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