DECIPHERING NK CELL TACTICS IN HIV-1: TRAIL'S MULTIFACETED FUNCTIONALITY AND KIR2DL/HLA-C MEDIATED IMMUNE PRESSURE

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Acronym Definition

ADCC	Antibody-dependent cell-mediated cytotoxicity
AIDS	Acquired immune deficiency syndrome
ART	Antiretroviral therapy
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
CLP	common lymphocyte progenitor
CMV	Cytomegalovirus
CTL	Cytotoxic T Lymphocytes
DcR	Decoy receptor
DD	Death domain
DED	Death effector domain
DISC	Death-inducing signaling complex
DNA	Desoxyribonucleic acid
DAP	DNAX activation protein
DR	Death receptor
FasL	Fas ligand
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
IFN-γ	Interferon γ
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activating motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
KIR	Killer-cell immunoglobulin like receptor
Lck	Lymphocyte-specific protein tyrosine kinase
LAMP	Lysosomal-associated membrane protein
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
mRNA	Messenger ribonucleic acid
NCR	Natural cytotoxicity receptor
Nef	Negative factor
NK	Natural killer

NKG2	Natural-killer group 2 receptors
NKP	NK cell progenitor
OPG	Osteoprotegerin
PBMC	Peripheral blood mononuclear cell
RNA	Ribonucleic acid
SIV	Simian immunodeficiency virus
Tat	Trans-activator
TNF-α	Tumor necrose factor α
TRAIL	Necrosis factor related apoptosis inducing ligand
ULBP	UL16 binding protein
Vif	Viral infectivity factor
Vpu	Viral protein u
ZAP70	Zeta-chain-associated protein kinase

Abstract

NK cells are critical components of the antiviral immune response, detecting changes in the cell surface composition of infected cells through a diverse receptor repertoire, including highly polymorphic killer cell immunoglobulin-like receptors (KIRs) and conserved effector molecules, shared by every individual. In my investigation, we explored the role of these receptors in the context of HIV-1 infection.

Through a large-scale screening approach combined with functional assays, we identified Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL) to be associated with NK cell degranulation against HIV-1-infected cells. Exploring TRAIL's mechanisms, we uncovered its multifunctionality, extending beyond its known role in inducing receptor-mediated cytotoxicity. Our findings suggest that direct stimulation of membrane-bound TRAIL, through death receptors, triggers degranulation and IFN-γ release. Additionally, we observed that Decoy Receptor 1 (DcR1) and Osteoprotegerin (OPG) induce degranulation, challenging previous notions of their protective roles in TRAIL-mediated cytotoxicity. Based on these discoveries, we suggest that TRAIL endows a multifaceted role in HIV-1 infection, orchestrating various effector functions beyond its conventional role in apoptosis induction via receptor-mediated pathways.

Contrary to the conserved molecule TRAIL, we focused in a separate investigation on the highly polymorphic inhibitory KIR2DL receptors due to emerging evidence of HIV-1's differential modulation of their cognate binding partner, HLA-C. Accumulating evidence indicate that HLA-C/KIR2DL interactions can drive HIV-1-mediated immune evasion and contributes to the intrinsic control of HIV-1 infection. Therefore, we examined the impact of specific KIR/HLA-C binding affinities on NK cell receptor repertoire, phenotypically analyzed NK cells, and sequenced HLA-C-modulating viral protein, Vpu, in 122 viremic, untreated HIV-1+ individuals. Our results revealed a genotype-dependent expansion of KIR2DL1+ NK cells, correlated with increased binding affinities between KIR2DL1 and HLA-C allotypes, suggesting that HIV-1 influences NK cell subset composition in a KIR-dependent manner. Furthermore, we observed a preferential selection of Vpu variants that downregulate HLA-C with higher KIR2DL/HLA-C binding affinities, indicating that HIV-1 evades immune responses based on host genetics. This underscores the ongoing arms race between NK cells and HIV-1, highlighting the sophisticated strategies employed by the virus to evade immune surveillance and the importance of host genetic factors in shaping the immune response to HIV-1 infection.

1 Introduction

1.1 Innate immunity

The human body is consistently exposed to potential threats in form of invading pathogens or malignant transformed cells. To effectively counter these hazards, humans have evolved a highly specific and rapid protective system. This intricate defense system involves a coordinated interplay of cellular and humoral responses and is primarily characterized by two essential components (Chaplin 2010). The adaptive immune response relies on specific lymphocytes, exhibiting a remarkable specificity towards particular antigens. It also encompasses a memory function, providing protection against recurring infections. Although the adaptive response ensures flexible and precise recognition of dangerous antigens, this process requires several days. In contrast, innate immunity complements the adaptive response by mounting an immediate reaction to pathogens. This rapid response is executed through a pre-programmed repertoire of biochemical agents, including inflammatory cytokines and complement proteins, along with specialized immune cells (Beutler 2004).

1.2 NK cells

Natural killer (NK) cells are specialized effector lymphocytes that form a crucial part of the innate immune response. Discovered in 1975 by Kiessling, Herberman and their colleagues, these lymphoid cells were found to possess intrinsic lytic activity against tumor cells without the need for prior immunization with alloantigens or tumor-associated antigens (Herberman et al. 1975; Kiessling et al. 1975). NK cells exhibit a distinctive granular morphology and constitute a range of 5% to 15% among lymphocytes in human peripheral blood (Azargoon et al. 2019). Beyond circulating in peripheral blood, NK cells can be found in various tissues such as the liver, uterus, spleen, and lungs, and to a lesser extent, in secondary lymphoid tissues (Dogra et al. 2020). The “linear” model proposes that NK cells originate from CD34+ hematopoietic progenitor cells in the bone marrow and progress through common lymphocyte progenitors (CLP) (Freud et al. 2005). CLPs gradually downregulate CD34 while upregulating CD127 and CD7 (Miller et al. 1994). However, the linear model is argued since it cannot explain the vast diversity in NK cell populations (Cichocki et al. 2019). Environmental factors, such different cytokine milieu, can cause plastic hematopoietic progenitor cells to favor different pathways. Previous findings evidently show that NK cells can also be derived from common myeloid precursor (CMP) when cultured with NK cell-supporting cytokines (Grzywacz et al. 2011). According to the prevailing linear model, CLPs transition into CD122+ Pre-T/early NK

cell progenitor (NKP) lineages and the expression of CD56 by CD122+ NKPs marks their fate as committed immature NK cells. The emerging CD56+ subset is identified as CD56bright cells, which further mature into CD56dim NK cells and is accompanied by the expression of the immunoglobulin superfamily member CD16 (FcγRIII) (Yu et al. 2013). This transition is based on *in vitro* studies of NK cell development from hematopoietic progenitor cells and the shorter length of telomere in CD56dim NK cells (Chan et al. 2007). The final stage of NK cell ontogeny introduces terminally differentiated NK cells expressing CD57 (Abel et al. 2018). CD57 serves as a marker for highly differentiated NK cells, as its expression is associated with replicative senescence (Brenchley et al. 2003). While absent in fetal NK cells, observations show an increase in CD57 expression with age (Le Garff-Tavernier et al. 2010; Abo et al. 1984). Björkström et al. observed that NK cells expressing CD57 exhibit diminished responsiveness to cytokine stimulation and a reduced proliferative capacity (Björkström et al. 2010).

1.2.1 NK cell receptors

NK cells play a pivotal role in the immediate defense against abnormal transformed cells or pathogens as part of the first line of defense. Their ability to discriminate between friend or foe is facilitated by an intricate array of activating and inhibitory receptors (Lanier 2005). Despite exclusively carrying germline-encoded receptors, these receptors exhibit significant differential expression, resulting in remarkable diversity with up to 100,000 different phenotypes (Horowitz et al. 2013). This diverse set of receptors enables the integration of signals received from target cells, thereby eliciting a corresponding response. The maintenance of homeostasis and the distinction between self and non-self-antigens heavily depends on Human Leukocyte Antigen class I (HLA-I) molecules present on the surface of all nucleated cells (Natarajan et al. 2002; Kärre et al. 1986). HLA-I molecules induce a robust inhibitory signal, ensuring NK cells remain quiescent, as sporadic activating signals alone are insufficient to outweigh the inhibitory signal, thus achieving self-tolerance (**Fig. 1A**) (Raulet und Vance 2006).

In the context of viral infection or transformed malignant cells, the delicate balance of inhibitory and activating ligands on target cells is disrupted. This disruption can occur through the excessive expression of activating ligands, overriding the inhibitory signal. As a result, NK cells are triggered and eliminate the target cell; a phenomenon known as induced-self (Bauer et al. 1999). Alternatively, in certain tumor cells or virally infected cells, a downregulation, particularly of HLA-I molecules, in inhibitory ligands is provoked. This downregulation also leads to activation and subsequent elimination of target cells, a process termed missing-self (Kärre et al. 1986). Of note, tumor cells and viruses employ the strategy of HLA-I downregulation to evade T cell recognition (Koutsakos et al. 2019). The finely tuned response to missing-self is

achieved through a process known as NK cell education, crucial for the development of fully functional NK cells (**Fig. 1B**) (Kim et al. 2005; Anfossi et al. 2006). Current literature emphasizes that NK cells express at least one inhibitory receptor, and it must engage with its cognate self-HLA-I molecule during NK cell development. The effector function of NK cells is tuned by the affinity of these inhibitory interactions referred to as Rheostat Model (Brodin et al. 2009). The introduction of an education process stems from the observation that NK cells lacking inhibitory receptors exhibit hyporesponsiveness against HLA-I-deficient target cells (Anfossi et al. 2006).

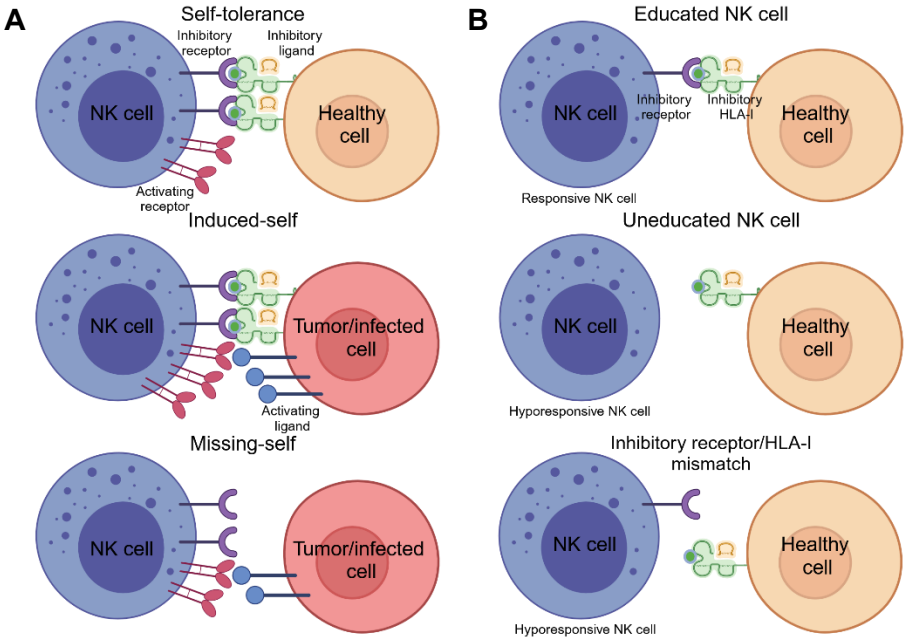

Figure 1: Regulation of NK cell activity and education.

A *In healthy cells, high levels of inhibitory NK cell ligands are expressed, maintaining NK cells in a quiescent state. Aberrant cells can upregulate activating ligands, overriding the inhibitory signals, leading to NK cell activation (induced-self). Abnormal cells downmodulate inhibitory signals resulting in NK cell activation (missing-self).* **B** *During development, NK cells undergo a process of education in which they must engage with inhibitory ligands to attain functional competence. NK cells become hyporesponsive (uneducated) when they fail to express inhibitory receptors or when an HLA-I/inhibitory receptor mismatch is present.*

Receptors with the ability to engage HLA-I molecules are the killer immunoglobulin-like receptors (KIR) (Wagtmann et al. 1995; Moretta et al. 1996). The KIR gene family exhibits significant diversity between individuals due to allelic polymorphism, copy number variations, and the presence or absence of KIR genes (Shilling et al. 2002; Uhrberg et al. 1997). KIR genes can encode for inhibitory or activating receptors, with KIRs being type I transmembrane proteins featuring either a long (L) or short (S) cytoplasmic tail. Corresponding to their

cytoplasmic tail, KIRs are either inhibitory with immunoreceptor tyrosine-based inhibition motifs (ITIMs) or activating with an immunoreceptor tyrosine-based activating motif (ITAM) (**Fig. 2**). KIRs recognize distinctive HLA-C, HLA-B and very few HLA-A epitopes, which can serve as activating or inhibitory ligands (Vilches und Parham 2002).

Another family of NK cell receptors is the C-type lectin family. The prominent inhibitory receptor of this family is NKG2A, which forms a heterodimer with CD94 through disulfide bonds and transmits its inhibitory signal through its ITIM motif (Lazetic et al. 1996). In contrast to the KIR gene family, NKG2A exhibits low genetic diversity, and its binding partner, the non-classical HLA-I molecule HLA-E, is characterized by limited polymorphism (Braud et al. 1998). Activating members of this family include the heterodimer NKG2C/CD94 and the homodimer NKG2D. NKG2C co-localizes with the ITAM-containing molecule DNAX activation protein 12 (DAP12) and also binds to HLA-E (Lanier et al. 1998a). NKG2D is associated with DAP10, which bears the YINM motif as an activation domain. Ligands for NKG2D, unlike HLA-E, are not constantly expressed on target cells but can be induced by cellular stress such as DNA damage, cellular transformation, or infection (Lanier 2015).

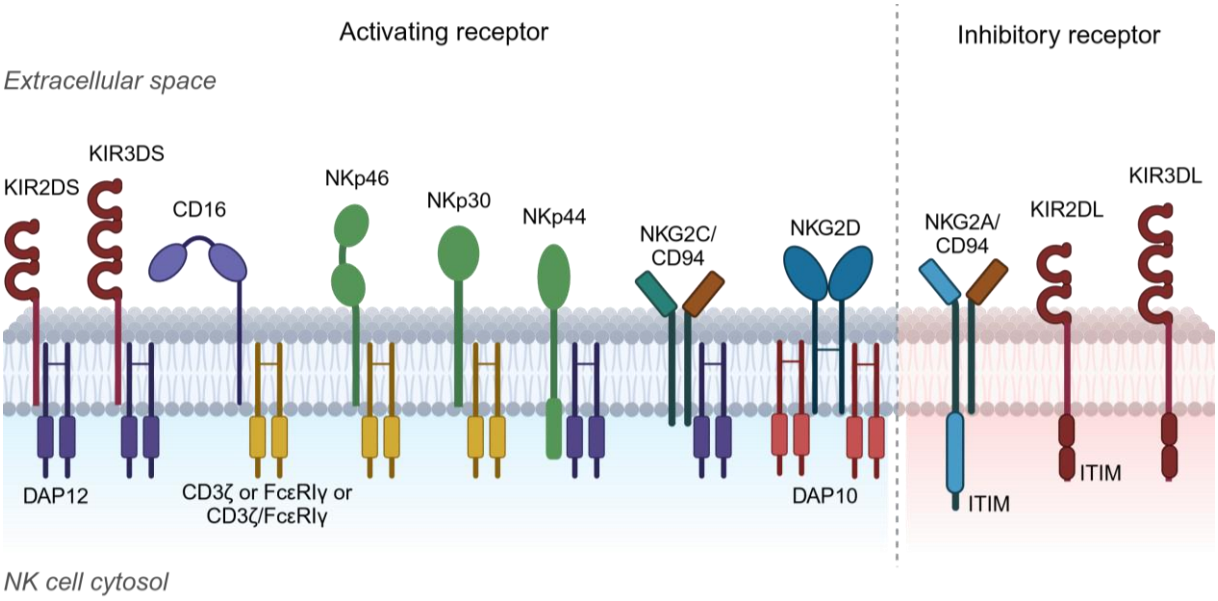

Figure 2: Activating and Inhibiting NK cell receptors

The Illustration depicts activating (left) and inhibitory (right) receptors of NK cells. Activating receptors include activating KIRs (e.g., KIR2DS, KIR3DS), CD16, members of the NCR family (e.g., NKp46, NKp30, NKp44), and members of the C-lectin like family (e.g., NKG2C, NKG2D). These receptors recruit various adaptor molecules (such as DAP10, DAP12, CD3ζ, or FcεRIγ), which contain activation motifs, thereby triggering activating signals. Inhibitory receptors comprise inhibitory KIRs (e.g., KIR2DL, KIR3DL) and the C-lectin like family member NKG2A. Inhibitory signals are transmitted through cytosolic ITIMs.

Additional important activating receptors can be found in the natural cytotoxicity receptor (NCR) family, which includes NKp46, NKp44, and NKp30. NCRs are proposed to bind to various cellular ligands implicated in NK cell surveillance of virally infected or tumor cells (Barrow et al. 2019). NKp46 and NKp30 are constitutively expressed on NK cells, and binding results in signal transmission via a CD3ζ - or FcεRIγ-domain containing multiple ITAM motifs (Pende et al. 1999; Sivori et al. 1997). In contrast, NKp44 in peripheral blood NK cells can only be induced by cytokine exposure. Binding of NKp44 leads to signal transduction using the DAP12 adapter molecule (Vitale et al. 1998).

In addition to using CD16 as an NK cell marker, its engagement induces a robust activation stimulus. CD16+ NK cells are recruited to cells with bound antibodies, where the constant region of an immunoglobulin G (IgG) antibody (Fc-region) serves as a ligand for CD16, mediating a response named antibody-dependent cellular cytotoxicity (ADCC) (Lanier 1998; Lanier et al. 1986).

In summary, NK cells utilize their sophisticated array of receptors to receive inhibitory and activating signals from target cells. If this input surpasses the threshold for NK cell activation, NK cells exert their effector functions.

1.2.2 Killer-cell Immunoglobulin-like Receptors

The killer immunoglobulin-like receptors (KIR) are encoded on chromosome 19q13.4 within the leukocyte receptor complex (LRC), encompassing 15 gene loci: *KIR2DL1*, *KIR2DL2/L3*, *KIR2DL4*, *KIR2DL5A*, *KIR2DL5B*, *KIR2SD1*, *KIR2DS2*, *KIR2DS3*, *KIR2DS4*, *KIR2DS5*, *KIR3DL1/S1*, *KIR3DL2*, *KIR3DL3* and two pseudogenes, *KIR2DP1* and *KIR3DP1* (Wilson et al. 2000). The current nomenclature is based on the number of Ig-like extracellular domains, categorized as either three domains (3D) or two domains (2D), and the length of their cytoplasmic tail, which can be short or long (Marsh et al. 2003). Two broad haplotypes have been identified: group A, predominantly expressing inhibitory receptors, and group B, featuring more activating KIRs with at least one additional gene not represented in group A (**Fig. 3**) (Uhrberg et al. 1997). The capacity to function as activating KIRs is conferred by charged lysine residues in their transmembrane region, enabling the recruitment of the adapter protein DAP12 containing an ITAM motif in its cytoplasmic tail (Lanier et al. 1998b). In contrast, inhibitory KIRs carry ITIM motifs in their cytoplasmic tail, resulting in phosphate binding and the exertion of inhibitory signals (Burshtyn et al. 1996).

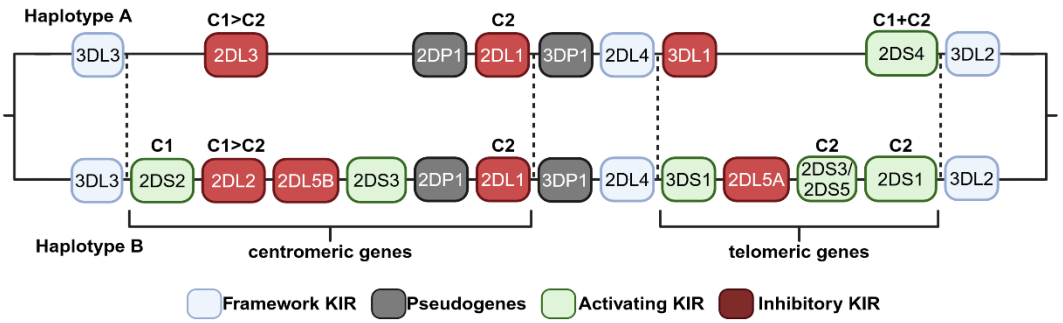

Figure 3: KIR haplotype

15 KIR genes and 2 pseudogenes have been identified, organized into haplotype A and haplotype B. These haplotypes comprise various activating (green) and inhibitory (red) KIR gene loci, categorized into centromeric and telomeric genes. Framework genes (light blue), present in every individual, encompass the KIR-expressing genes. Pseudogenes (grey) are also present in every individual. Above certain KIRs, the binding epitope of HLA-C is depicted (C1 or C2).

KIR3D receptors specifically recognize a variable sequence of HLA-B in the 77-83 amino acid region. Depending on the sequence, it can exclusively form the Bw4 or Bw6 epitope. However, only the Bw4 motif interacts with KIR3D receptors (Gumperz et al. 1995). This motif is also present in certain HLA-A alleles, such as *A23*, *A24*, *A25*, and *A32*, enabling them to inhibit KIR3DL+ NK cells (Foley et al. 2008). Whereas, KIR2D is binding HLA-C molecules, depending on the allotype that is stratified into the C1 epitope (asparagine at position 80) and C2 epitope (lysine at position 80) (Mandelboim et al. 1996).

1.3 HLA-I molecules

Expressed on all nucleated cells, Human Leukocyte Antigen class I (HLA-I) molecules are key regulators in the immune response of the human body, exhibiting significant allele polymorphism with 26,341 different alleles (Robinson et al. 2015).

The HLA-I gene locus is embedded in the chromosomal position 6p21 that are organized into two major cluster encoding for HLA-I and HLA-II molecules. The HLA-I locus comprises genes encoding classical (HLA-A, -B, and -C) and non-classical (HLA-E, -F, and -G), while HLA-II encodes for HLA-DP, -DQ, and –DR (Complete sequence and gene map of a human major histocompatibility complex. The MHC sequencing consortium 1999). Both classical and non-classical HLA-I molecules play a crucial role in NK cell biology as cognate binding partners for

NK cell receptors. Conversely, only specific *HLA-DP* alleles have recently been demonstrated to bind to NK cells (Niehrs et al. 2019). The HLA-I molecule consists of a polymorphic glycoprotein heavy chain, containing α1, α2, and α3 domains, in association with β2m-microglobulin, a small soluble protein (Saper et al. 1991). These molecules present intracellular-derived peptides, typically in the 8–10 amino acid residue range, although there are non-classical HLA-I molecules that bind longer peptides or none at all (Dulberger et al. 2017; Garcia-Beltran et al. 2016; Arosa et al. 2007). The accumulation of allelic polymorphism near the peptide binding groove results in vast diversity in peptide presentation (Ilca et al. 2019). Proteins tagged with ubiquitin are degraded in the cytosol by proteosomes and translocated into the endoplasmic reticulum (ER). This process is catalyzed by transporters associated with antigen processing (TAP) TAP1:TAP2 heterodimers. In the ER lumen, the peptide loading complex (PLC), comprising HLA-I molecules, TAP, tapasin (also known as TAP-binding protein (TAPBP)), ERp57, and associated calreticulin and calnexin, facilitates the loading of peptides onto HLA-I. Tapasin aids interaction of TAP with HLA-I α-chain that have yet to receive peptides. ERp57, calreticulin and calnexin ensures proper folding of high-affinity peptides and loading them onto HLA-I molecules. The finalized HLA:β2m complex is released form the ER and shuttled through the Golgi apparatus to the cell surface (Neefjes et al. 2011).

This mechanism of intracellular peptide presentation reflects the intracellular proteome at the cell surface. Healthy cells display self-peptides and self-tolerance is mediated. In the case of viral infection, replicating viruses inside cells contribute to the cellular proteome. These virus-derived peptides are also sampled on the surface and recognized by CD8 T cells (Hansen et al. 2009). Whereas NK cells sense alteration in HLA-I expression or virus-derived peptides effect high affinity binding of KIRs and thus triggering NK cell effector functions.

1.3.1 HLA-C

HLA-C has been highlighted to play a pivotal role in immune response against pathogens, in cancer, the rejection or acceptance of organs and tissues and preeclampsia in pregnant women (Hiby et al. 2004; Velardi 2008; Wiśniewski et al. 2012). This importance is based on the communication between HLA-C and its cognate binding partner KIR.

It has emerged as most recently evolved member among classical HLA-I molecules that is only present in humans and great aps. The discovery of *HLA-C* led to the hypothesis of a non-functional gene, since HLA-C restricted T cells have not been described at this time (Güssow et al. 1987). With the introduction of the KIR family, HLA-C serving as primarily ligand for KIR has emerged. In contrast to HLA-A and -B, HLA-C surface expression is significantly lower than HLA-A and HLA-B, which also supports its role as a regulated marker than an overall

sampling and presenting the intracellular proteome (Apps et al. 2015). *HLA-C* locus displays numerous subtle changes, which affect its regulation. Gene elements in the proximal and distal promoter region include an interferon-stimulated response element (IRSE) that is able to recruit interferon response factor (IRF) to induce transcription. A process that is initiated by cytokines, such as interferon-γ (IFN-γ) (Johnson 2003). Another essential protein affected by IFN-γ that regulates HLA-C expression is NOD-like receptor caspase recruitment domain containing protein 5 (NLRC5). Studies showed that HLA-I transcription was facilitated by specific recruitment of NLRC5 to the SXY module upstream of the HLA transcription site (Kobayashi and van den Elsen 2012). As an additional regulating element, *HLA-C* locus contains an EnhancerA region that is shared in each HLA-I locus (van den Elsen et al. 1998). However, due to mutated binding sites for the multi-functional transcription factor NF-κB in this *HLA-C* element, regulation of *HLA-C* by cytokines is less sensitive compared to *HLA-A* and *HLA-B* (Gobin et al. 1998; Johnson und Pober 1994). As a primary ligand for KIR, HLA-C allotypes bind to four activating KIRs (KIR2DS1, KIR2DS2, KIR2DS4, and KIR2DS5) and three inhibitory KIRs (KIR2DL1, KIR2DL2, and KIR2DL3). The specificity of HLA-C for KIR2DL1, KIR2DL2, or KIR2DL3 is determined by dimorphisms at positions 77 and 80 located on the α1-helix of the antigen-binding cleft. Crucial for this binding is position 80, where an asparagine (C1 motif) defines its specificity to KIR2DL2 and KIR2DL3, while lysine at that position favors binding of KIR2DL1 (C2 motif) (Winter et al. 1998). The HLA-C2 motif is able to engage exclusively KIR2DL1, KIR2DS1 and some KIR2DS5 allotypes that carry a methionine at position 44 (Rajagopalan und Long 1997; Stewart et al. 2005; Blokhuis et al. 2017). Whereas the HLA-C1 epitope interacts with KIR2DL2 and KIR2DL3 and some KIR2DS2 allotypes carry a lysine at position 44 (Moesta und Parham 2012). Winter and Long demonstrated that a single amino acid change from methionine at position 44 in KIR2DL1 to lysine, present in KIR2DL2 and KIR2DL3 alters the binding specificity towards HLA-C motifs and vice versa (Winter und Long 1997). While KIR2DS4 displays a cross-reactivity to both epitopes and binds the single HLA-A allotype **1102* (Graef et al. 2009). Notably, certain KIR2DL2 and KIR2DL3 allotypes show a dual binding affinity to the HLA-C1 and -C2 motif with a higher specificity to HLA-C1 epitopes (Winter und Long 1997). KIR2DL1 and KIR2DL2/3 can be further stratified into three and four distinctive phylogenetic clades, respectively, based on 26 KIR2DL1 and 36 KIR2DL2/3 coding sequences. Clade 1 of KIR2DL1 consists of *KIR2DL1*001*-like alleles, clade 2 of *KIR2DL1*003*-like alleles, and clade 3 of *KIR2DL1*004*-like alleles. For KIR2DL2/3, clade 1 and 2 include *KIR2DL3* alleles, while clade 3 and 4 comprise *KIR2DL2* alleles. Previous studies have shown that KIR2DL1 on haplotype A binds with greater avidity for C2 motifs compared to haplotype B (Hilton et al. 2015). Conversely, higher avidity of KIR2DL2/3 for C1 was associated with haplotype B.

1.4 Effector functions

1.4.1 Degranulation

NK cells diligently survey their environment for potential threats, initiating a cascade of events upon sensing a target that culminates in the formation of an immunological synapse. The establishment of conjugates is facilitated by the interaction of adhesion molecules, ensuring a tight coupling to target cells. As NK cells scan the surface of the target cell with their diverse receptor repertoire, the activation signal, when outweighing the inhibitory input, triggers a process known as degranulation (**Fig.4**).

Upon engagement of activating receptors, clustering occurs, leading to the recruitment of protein kinases. These kinases phosphorylate ITAMs in the cytoplasmic tail, subsequently recruiting and activating Syk and ZAP70 tyrosine kinases (Lanier et al. 1998b; Brumbaugh et al. 1997). In contrast to ITAM motifs, DAP10, which carries the YINM motif, associates with Grb2 and the effector molecule Vav1 and the p85 subunit of phosphoinositide 3-kinase (PI3K) (Upshaw und Leibson 2006). Recruitment of activated kinases initiates a cascade of downstream phosphorylation events involving signaling molecules such as PI3K, phospholipase C (PLC)-c1 and -c2, and guanine nucleotide exchange factors Vav-1, 2, 3. Phosphorylation of signaling molecules is accompanied by a calcium influx and the initiation of the MAPK signaling pathway (Meza Guzman et al. 2020). Consequently, a reorganization of the actin cytoskeleton is triggered. The microtubule organizing center (MTOC) polarizes towards the immunological synapse, guiding cytotoxic granules along microtubules towards the synapse (Orange et al. 2003; Clark und Griffiths 2003).

These cytotoxic granules release their content, primarily composed of granzyme B and perforin, within the immunological synapse. Perforin polymerizes at the target cell membrane, forming pores that granzyme B utilizes to diffuse into the target cell cytosol. Once inside, granzyme B cleaves caspase 3, initiating target cell apoptosis (Metkar et al. 2003). To protect themselves from the lytic components released into the immunological cleft, NK cells present the protein Lysosomal-Associated Membrane Protein 1 (LAMP-1), also known as CD107a, on their inner membrane of lytic granules. Membrane fusion and subsequent exocytosis of cytotoxic granules result in the presentation of CD107a on the outer membrane. CD107a surface expression reduces the binding of perforin, thereby protecting NK cells (Cohnen et al. 2013). This phenomenon serves as a marker for the exocytosis of cytolytic granules by NK cells and thus as a surrogate marker for NK cell killing (Alter et al. 2004).

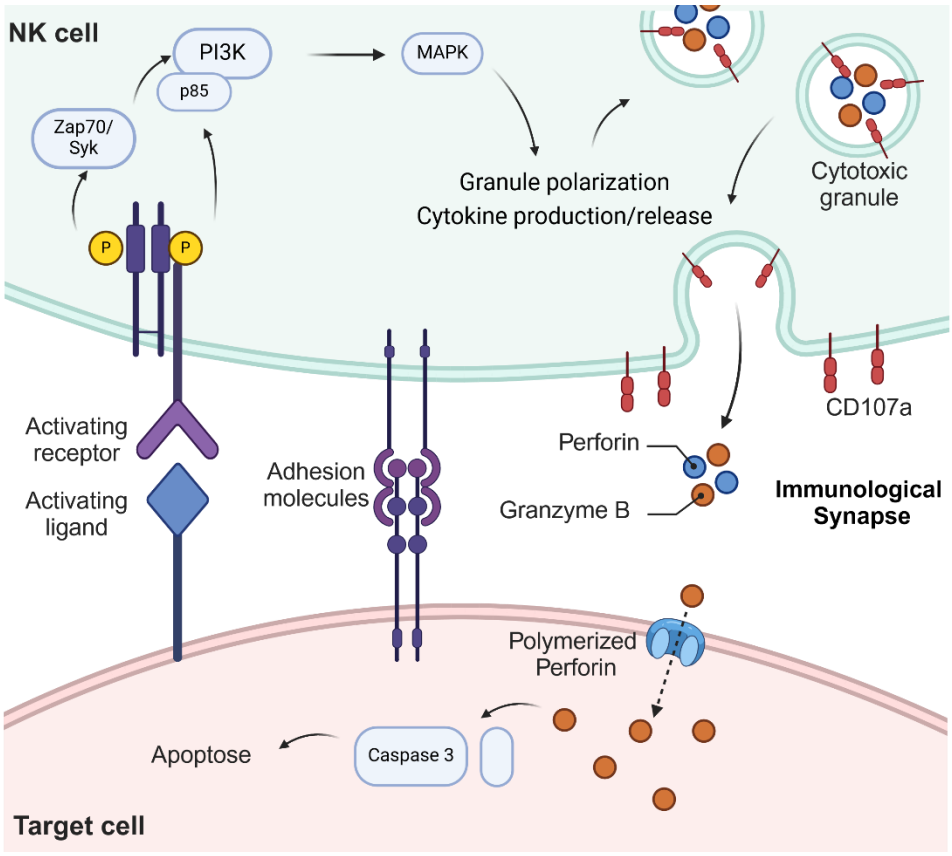

Figure 4: Immunological synapse

The illustration depicts a simplification of an immunological synapse and subsequent signal transduction. NK cells attach to target cells through the engagement of adhesion molecules and scan the surface with their diverse set of receptors. Binding to an activating ligand induces the phosphorylation of adapter proteins. Depending on the adapter protein, certain kinases are subsequently activated. For example, DAP10 associates with p85, while DAP12 associates with Syk or Zap70. Phosphorylation cascade is initiated, leading to the activation of the PI3K followed by MAPK signaling pathway activation. As a result, cytokines are produced, and cytotoxic granules are released into the immunological synapse. Due to exocytosis of cytotoxic granules, CD107a is presented on the NK cell surface and protects the cell from perforin. Perforin polymerizes at the target cell membrane, forming pores. Granzyme B utilizes these pores to diffuse into the target cell, where it cleaves caspase 3, initiating a downstream signaling pathway that leads to apoptosis.

1.4.2 Receptor mediated cytotoxicity

In addition to degranulation-mediated killing, NK cells possess another mechanism to induce cytotoxicity, known as receptor-mediated cytotoxicity. Proteins involved in this process, belonging to the TNF-Superfamily, are expressed on NK cells and include Fas Ligand (FasL),

Tumor Necrosis Factor (TNF), and Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL) (Zhou et al. 2017). Within this family, TRAIL has been demonstrated to be pivotal in viral infections (Sag et al. 2019). TRAIL is a type II transmembrane protein with a single transmembrane domain, an extracellular C-terminus, and an amino-terminal end located in the cytosol (Cha et al. 1999). Functional TRAIL can exist in membrane-anchored form, as well as a soluble form due to protease-mediated cleavage or being coupled to extracellular vesicles shed by cells (Ehrlich et al. 2003; Monleón et al. 2001). Five different binding partners for TRAIL have been identified, including four membrane-bound receptors: Death Receptor 4 (DR4), Death Receptor 5 (DR5), Decoy Receptor 1 (DcR1), and Decoy Receptor 2 (DcR2). TRAIL also interacts with the soluble factor Osteoprotegerin (OPG) (Degli-Esposti et al. 1997a; Degli-Esposti et al. 1997b; Emery et al. 1998; Pan et al. 1997a; Pan et al. 1997b).

TRAIL expression is inducible and serves as an effective tool for innate and adaptive immune cells to control infections and elicit appropriate immune responses. The activation of the immune system leads to the systemic release of type-I interferons, such as IFN-α and IFN-β, which induce TRAIL expression on T cells, dendritic cells, monocytes, and NK cells (Fanger et al. 1999; Cardoso Alves et al. 2021; Griffith et al. 1999). Additionally, IL-2 and IL-15 have been shown to upregulate TRAIL expression on NK cells (Kayagaki et al. 1999b). Coinciding, TRAIL receptors on infected or malignant cells are upregulated, rendering them susceptible to TRAIL-mediated apoptosis (Brincks et al. 2008; Clarke et al. 2000; Kotelkin et al. 2003). Next to TRAIL receptor-mediated cytotoxicity, recent studies in mice by Alves et al. demonstrated that TRAIL receptor binding on NK cells promotes IL-15-dependent granzyme B production (Cardoso Alves et al. 2020).

Depending on the targeted receptor, different signals are induced. TRAIL is famously known to induce apoptosis through the coordinated cell death process transduced by DR4 and DR5. TRAIL trimerizes and engages the resulting cluster of death receptors, recruiting the adaptor FAS receptor-associated protein with the death domain (FADD) to the cytosolic death domain (DD). Subsequently, the death effector domain (DED) exposed in FADD engages procaspase-8 and procaspase-10, forming the death-inducing signaling complex (DISC). The proximity of pro-caspases 8 and 10 facilitates their autoactivation by cleavage and subsequent release into the cytosol. There, they cleave downstream effector caspases such as caspase 3, initiating an effector caspase cascade that ultimately leads to cell death (Falschlehner et al. 2007; Schneider et al. 1997). Moreover, TRAIL has been shown in previous studies to induce the activation of NF-κB, ERK1/2, p38, and PI3K-AKT in tumor cells, facilitating processes such as migration, proliferation, and invasion (Azijli et al. 2013). Conversely, DcR1 and DcR2 do not instigate apoptosis upon TRAIL ligation. DcR1, anchored by glycosylphosphatidylinositol in the cell membrane, lacks transmembrane and death domains, while DcR2 possesses a truncated

cytosolic domain, thus failing to transmit a signal. Finally, engagement of TRAIL by OPG sequesters its functional competence to induce apoptosis (Jong et al. 2022).

1.4.3 Cytokines

Stimulation of NK cells through target cell engagement or exposure to cytokines triggers the release of a diverse array of chemokines, including CCL3, CCL4, and CCL5, as well as effector cytokines such as IFN-γ, TNF, GM-CSF, IL-10, IL-5, and IL-13. These effector molecules play pivotal roles in immunity, acting as signaling mediators that modulate cellular responses within both the adaptive and innate immune systems (Paul und Lal 2017).

Among these molecules, IFN-γ stands out for its potent antiviral activity. On the one hand, it endows an immunomodulatory function by augmenting antiviral activity of immune cells (Saha et al. 2010). On the other hand, it exerts direct protective effects by disrupting viral entry, replication, and gene expression (Kang et al. 2018). Additionally, IFN-γ stimulates cells to modify the expression of caspase, FAS ligand, and TRAIL, all of which contribute significantly to the antitumor response (Kaplan et al. 1998). The multifaceted actions of these effector molecules highlight their crucial functions in orchestrating immune responses against various challenges.

1.5 Human Immunodeficiency Virus (HIV)

Human Immunodeficiency Virus (HIV) is identified as the causative retrovirus that induces Acquired Immunodeficiency Syndrome (AIDS) (Barré-Sinoussi et al. 1983). It can be categorized into two main subtypes: HIV-1 and HIV-2, with HIV-2 being characterized by lower transmission rate and reduced pathogenicity (Gilbert et al. 2003; Nyamweya et al. 2013). Phylogenetic data conclusively indicate that HIV-1 originates from its relative simian immunodeficiency virus (SIV) and was transmitted from chimpanzees to humans (Gao et al. 1999). HIV-1 is further divided into a major group (group M) and minor groups—groups N, O, and possibly a group P. Each group is suggested to represent an independent transmission event of SIV into humans (Keele et al. 2006). The major group M is further stratified into several clusters, each dominant in different areas of the world (Yamaguchi et al. 2020).

Over the last four decades, HIV-1 has infected an estimated 84 million people worldwide, resulting in approximately 40 million deaths (HIV 2024). The introduction of antiretroviral therapy (ART) marked a significant turning point in HIV treatment. This therapy, consisting of different drugs targeting HIV entry, replication, and integration, successfully suppresses viral spread and transmission within the body. When introduced, ART prevents the onset of AIDS,

allowing individuals to lead normal life expectancies. Due to ART, numbers of AIDS- related deaths have declined, however access to therapy is not universal, and the prospects of curative treatments and an effective vaccine are elusive (Richman et al. 2009; Davenport et al. 2019; Barouch 2008).

AIDS is defined by a decrease in CD4 T cells below 200 cells per µL or the presence of distinctive AIDS-defining illnesses, reflecting an underlying immune deficiency. The acute phase of HIV-1 infection is characterized by the peak of HIV RNA (nadir) and a rapid decline of CD4 T cells (The Stages of HIV Infection | NIH 2024). The subsequent latent phase exhibits a short recovery of CD4 T cell counts as the immune system challenges the virus. However, this brief recovery is followed by a continuous decline in CD4 T cells, accompanied by an increase in HIV RNA. This trend progresses into the onset of AIDS, characterized by a CD4 T cell count below 200 cells/µL. At this stage, the individual is severely immunocompromised, displaying a high susceptibility to opportunistic infections and a heightened mortality risk.

1.5.1 HIV-1 replication cycle

HIV, classified as a human-specific lentivirus, belongs to the Retroviridae family. Members of this family are enveloped and contain positive-sensed single-stranded RNA. HIV carries two identical copies of single-stranded RNA with bound viral proteins crucial for the development of a functional virion. The HIV genome, along with attached proteins, is enclosed by a conical capsid composed of the viral Gag protein p24. This capsid is embedded into a matrix, surrounded by a lipid membrane that anchors the glycoprotein gp120.

The entry of HIV virions is mediated by its glycoprotein 120. Gp120 binds to the highly expressed CD4 receptor on CD4 T cells (Maddon et al. 1986). For a successful fusion event, a second engagement of a co-receptor is necessary. Depending on the tropism of the HIV virus, it can either use CCR5 (R5-tropic) or CXCR4 (X4-tropic) as a co-receptor (Dean et al. 1996; Feng et al. 1996). Strains that can bind to both co-receptors are referred to as dual tropic. Subsequent conformational changes induce the fusion of the virion with the cell membrane. Viral compounds are released into the cytosol, and reverse transcription is initiated (Grove und Marsh 2011). Once reverse transcription is complete, the final viral double-stranded DNA is bound by viral integrase and translocated into the nucleus, where it is stably inserted into the host genome. The inserted viral DNA is transcribed into several viral mRNAs, subsequently translated into protein by hijacking host transcription and translation machinery. Temporal separation of viral proteins to be translated is conferred by their splicing ability (Emery und Swanstrom 2021). Completely spliced RNA leads to generation of Tat, Rev and Nef mRNA, hence they appear in early stages of infection. Tat regulates genes transcription

and Rev is responsible for nuclear export (Arrigo und Chen 1991; Berkhout et al. 1989). Nef modulates protein expression at the cell surface and enhances infectivity of viral particles (Chowers et al. 1994; Hung et al. 2007). Partially spliced RNA encodes for accessory viral proteins Vif, Vpr, Vpu, and Env (**Tab. 1**). Among these proteins, Vpu has been demonstrated to play an important role in modulating host cells to evade immune responses (Khan und Geiger 2021). The protein is composed of three α-helices, where the N-terminal helix anchors the protein into the cell membrane, ER or Golgi apparatus. Helices 2 and 3 are located in the cytosol and connected by a loop containing two phosphorylated Serine residues (S52 and S56) (Federau et al. 1996). These post-translational modifications contribute to the binding of helix 3 to β-TrCP/ubiquitin ligase complexes. This association confers Vpu the ability to label proteins, such as CD4 or antiviral protein tetherin, for degradation (Margottin et al. 1998). Lastly, viral unspliced mRNA is exported into the cytosol, translating the viral genome, Gag and Pol precursors. The assembly of viral particles is a highly selective and coordinated process that occurs at the cell membrane. Viral components are packed into the viral particle, and the fully assembled virion is released into the extracellular space. Budded virions are inherently immature and non-infectious. Therefore, an activation step is needed that requires cleavage of Gag and Gag-Pol polyproteins into their mature form (Sundquist und Kräusslich 2012).

***Table 1**: HIV-1 accessory viral proteins*

Protein	Function	Reference
Vpu (viral protein u)	CD4 and tetherin degradation, HLA-C downregulation, facilitates virus release, inhibition of NF-kb signaling	Apps et al. 2016; Douglas et al. 2009; Klimkait et al. 1990; Langer et al. 2019
Vif (viral infectivity protein)	Antiviral APOBEC3 degradation, promotes viral replication	Stopak et al. 2003; Chowdhury et al. 1996
Nef (negative regulating factor)	Enhance infectivity, downregulate CD4 and HLA-A and -B	Chaudhuri et al. 2007; Chowers et al. 1994; Hung et al. 2007
Vpr (viral protein r)	Enhances viral replication	Sato et al. 2013

1.5.2 NK cells in HIV-1 infection

NK cells play a crucial role in mounting immediate and robust responses against HIV-1 infection, significantly influencing the disease's progression and outcome. The immune responses by NK cells during HIV-1 infection can be categorized into generic responses and responses highly influenced by an individual's genotype.

In the generic response, CD16-positive NK cells recognize antibody-opsonized CD4 T cells, leading to ADCC-mediated elimination of infected cells (Wren et al. 2013; Ahmad et al. 2001a). Another important activating receptor in HIV-1 infection is NKG2D. The viral protein Vpr upregulates NKG2D-binding partner, UL16 binding proteins (ULBPs), making infected cells more susceptible to NK cell-mediated killing. However, the viral protein Nef counteracts this mechanism by reducing surface levels of NKG2D ligands to dampen NK cell activity (Richard et al. 2010; Ward et al. 2007; Cerboni et al. 2007). To limit NK cell response, multiple accessory viral proteins modulate infected cells to suppress the expression of activating ligands, such as those for NCRs (Mavilio et al. 2003; Maria et al. 2003). Despite these evasion mechanisms, NK cell-secreted chemokines, including CCL3, CCL4, and CCL5, can partially control HIV-1 infection by binding to the viral co-receptor CCR5 and inhibiting viral entry (Quillay et al. 2016).

The outcome and progression to AIDS during HIV-1 infection vary depending on the individual's genotype. The effectiveness of NK cells in responding to HIV-1 infection is modified by their *KIR/HLA-I* genotypes. To evade recognition by cytotoxic T lymphocytes (CTLs), the viral protein Nef downregulates HLA-A and HLA-B, rendering them susceptible to KIR3DL1-educated NK cell killing (Le Gall et al. 1998; Boudreau et al. 2016; Collins et al. 1998). However, the notion that HIV-1 infection selectively downregulates HLA-A and HLA-B was revised when Apps et al. used primary HIV strains for infection. They could show Vpu`s ability to reduce HLA-C expression to different extents (Apps et al. 2016). Additional studies demonstrated that Vpu-mediated HLA-C suppression, makes infected cells susceptible to NK cell elimination, particularly when KIR2DL-educated NK cells sense subtle changes in HLA-I expression (Körner et al. 2017). *In vivo* and *in vitro* evidence supports the concept that specific KIR2DL2/HLA-C combinations effectively suppress HIV-1 replication (Lin et al. 2016). Moreover, studies by Kiani et al. clearly showed that stronger education of KIR2DL+ NK cells is directly linked to the magnitude of their response (Kiani et al. 2019). Potent NK cell education, based on high-affinity binding, enables a sensitive detection of alterations in the expression levels of their cognate HLA-C binding partner in autologous infected CD4 cells. Further reports document that HIV-1 is targeting KIR2DL+ NK cells (Hölzemer et al. 2015; Ziegler et al. 2020). For instance, Fadda and colleagues identified one p24 Gag peptide that not only can stabilize HLA-Cw*0102, but also inhibits the infected cells from KIR2DL2+ NK cell mediated lysis (Fadda et al. 2012). HLA-E, as another HLA-I molecule, is also targeted by HIV-1, although the effect of downregulation of HLA-E remains elusive (van Stigt Thans et al. 2019). NKG2A+ NK cells exhibit the strongest response against autologous HIV-infected CD4 T cells, supported by the observation that high HLA-E levels inhibit NKG2A+ NK cells anti-HIV activity (Ramsuran et al. 2018; Lisovsky et al. 2015). Additionally, KIR3DL1+ NK cells are educated by HLA-Bw4 with preferential binding to the Bw4*80I than the Bw4*80T allotype (Gumperz et al. 1997; Cella et al. 1994). Early reports by Martin et al. in comprehensive genotype studies

showed a significant protective effect of individuals carrying high-expressing *KIR3DL1* alleles in combination with *HLA-Bw4*80I* against disease progression (Martin et al. 2007).

1.6 Hypothesis and Aims

Numerous studies have revealed various strategies employed by viruses to evade immune responses. Among the key players in antiviral defense are NK cells, which possess the ability to detect alterations in infected cells. Despite their critical role, depletion of NK cells during primary SIV infection in rhesus monkeys showed no significant impact (Choi et al. 2008). In contrast, multiple human studies have demonstrated the capacity of NK cells to recognize HIV-1-infected cells, with specific genotypes of NK cell receptors being strongly associated with HIV-1 infection outcomes (Carrington et al. 2008; Fellay et al. 2007; Flores-Villanueva et al. 2001; Hölzemer et al. 2017; Martin et al. 2007; Martin et al. 2002), suggesting potential species-specific differences in immune responses. These findings underscore the importance of human NK cells in the context of HIV-1 infection. The ability of NK cells to identify infected cells is mediated by their diverse receptor repertoire. This repertoire includes germline-encoded, highly polymorphic KIRs, which exhibit varying antiviral effects depending on individual genetic backgrounds. Additionally, NK cells are equipped with conserved effector molecules shared across all individuals, enabling a universal capacity for viral recognition. This thesis explores both these aspects—individual variability in KIR-mediated responses and the role of conserved NK cell receptors—in the context of HIV-1 infection.

Previous investigations have demonstrated NK cells' ability to exert effector functions after exposure to HIV-1-infected cells, irrespective of individual genotypes (Vieillard et al. 2005; Tomescu et al. 2015; Garrido et al. 2018). Building on these findings, we hypothesized that an individual’s ability to mount an effective NK cell response against HIV-1 is shaped by two key factors: the genetic background of their HLA and KIR interactions, and the universal presence of conserved receptors that enable NK cells to recognize and respond to infected cells.

To explore this hypothesis, a comprehensive immunological screening of antigens on primary human NK cells was conducted to identify candidates conferring NK cells with the ability to release cytotoxic granules in response to HIV-1-infected cells. Notably, TRAIL emerged as a molecule that correlated with an enhanced NK cell response. Given that TRAIL has traditionally been associated with receptor-mediated cytotoxicity, this thesis investigates the underlying mechanisms of TRAIL's role in NK cell degranulation.

Several publications have underscored the importance of KIR/HLA-I interactions in HIV-1 infection and the progression to AIDS. Particularly, KIR2D/HLA-C interactions have gained

attention due to their highly polymorphic nature and recent findings regarding HIV-1 modulation of HLA-C expression. However, the intricate interplay between HLA-C/KIR2DL interactions, NK cell-mediated immune pressure, and HIV-1 immune escape remains inconclusively understood.

Our hypothesis proposes that binding affinities of matching KIR2D/HLA-C molecules directly influence HIV-1, selecting for quasispecies with a distinctive ability to modulate HLA-C expression. To test this, we determined binding affinities between specific KIR2D/HLA-C allotypes. Additionally, a viremic, therapy-naïve HIV-1+ cohort was utilized to characterize the NK cell receptor repertoire, with a focus on KIR2D receptors, and compared to HIV- individuals. Finally, this HIV+ cohort underwent next-generation sequencing (NGS) of Vpu with matched plasma and PBMC samples.

2 Discussion

The vast phenotypical diversity observed in NK cells underscores the complexity of their activity, which is tightly regulated by the unique receptor profiles present on each NK cell (Horowitz et al. 2013; Schwane et al. 2020). These receptors, polymorphic KIRs and conserved effector molecules present in all individuals, such as TRAIL, dictate the NK cell's ability to recognize and respond to HIV-1-infected cells. This thesis explores the intricate arms race between HIV-1 and NK cells from dual perspectives. The first study investigates TRAIL and demonstrates its contribution in the antiviral activity of NK cells against HIV-1-infected cells. Through our investigation into TRAIL's role in HIV-1 infection, we unveil its multifaceted functionality. Beyond its established role in inducing receptor-mediated cytotoxicity, TRAIL also exhibits the capacity to initiate the release of cytotoxic granules, thereby exerting anti-HIV-1 activity. Our findings are further validated by *in vitro* experiments, where we selectively examined TRAILs interaction with its binding partner, triggering NK cell degranulation and IFN-γ release.

The second publication illuminates the genotype-dependent interplay between HLA-C/KIR2DL interactions, NK cell-mediated immune pressure, and HIV-1 immune escape. While early studies identified Nef's ability to downregulate HLA-A and -B from the cell surface, the prevailing notion for some time was that HLA-C, as a NK-cell receptor ligand, remained unaltered by HIV-1 infection, thus evading NK cell recognition (Collins et al. 1998). However, a paradigm shift occurred with the use of primary HIV-1 strains for infection instead of lab-adapted strains like NL4-3. Vpu emerged as a key accessory protein capable of modulating HLA-C expression, with reports documenting variations among primary isolates in their ability to downregulate HLA-C (Apps et al. 2016). These findings were complemented by studies showing that the degree of HLA-C downregulation depends on the HLA-C genotype of the individual (Bachtel et al. 2018). This variation suggests a HIV-1 adaptation to modulate NK cell response. Considering that the education status of NK cells in individuals varies based on the strength of their genetically defined KIR/HLA-C pairing, we hypothesized that NK-cell immune pressure selects for HIV-1 quasispecies that exhibit distinct Vpu-mediated abilities to modulate HLA-C expression for viral escape. Our findings revealed a genotype-dependent expansion of KIR2DL1+ NK cells, associated with increased binding affinities between KIR2DL1 and HLA-C allotypes, supporting the notion that HIV-1 impacts NK cell subset composition in a KIR-dependent manner. Furthermore, our data indicate a preferential selection of Vpu variants that downregulate HLA-C in individuals with higher KIR2DL/HLA-C binding affinities, providing further evidence that HIV-1 evades immune responses in a manner determined by host genetics. This underscores the dynamic co-evolution between NK cells and HIV-1, highlighting the sophisticated strategies employed by the virus to evade immune surveillance and the importance of host genetic factors in shaping the immune response to HIV-1 infection.

Binding of KIR2D with HLA-C is highly dependent in the amino acid residue at position 80 in the a1 domain. While inhibitory KIR2DL1 receptors engages with the C2 epitope (lysine 80), inhibitory KIR2DL3 recognizes the C1 epitope (asparagine 80) and inhibitory KIR2DL2 principally recognizes C1 but also has some cross-reactivity with C2. The KIR locus, that includes genes for KIR2DL1, KIR2DL2, or KIR2DL3, comprises two groups of KIR haplotypes (A and B). Depending on the haplotype, *KIR2DL1* on A encodes stronger inhibitory receptors compared to KIR2DL1 receptors embedded in haplotype B. Whereas *KIR2DL2* on haplotype B are characterized by strong inhibitory C1 receptors and *KIR2DL3* on haplotype A are considered weak inhibitory C1 receptors.

Our cell-based approach to assess binding avidities confirms binding specificities for C2 (KIR2DL1) and C2 (KIR2DL2/3), with the known exception of KIR2DL1*022, which possesses a lysine at position 44, conferring specificity towards C1 motifs (Winter et al. 1998; Hilton et al. 2015). Consistent with previous reports, KIR2DL2 exhibits a greater breadth of binding across the HLA-C2 group than KIR2DL3, although overall greater avidity by KIR2DL2 was not confirmed, possibly due to the low representation of KIR2DL2 receptors used in this study (Moesta et al. 2008; Schönberg et al. 2011). The notion that KIR2DL3 has lower avidity and broader specificity than KIR2DL1 was recapitulated (Hilton et al. 2012). Hilton et al. reported higher avidities of KIR2DL1 for HLA-C2 in haplotype A and KIR2DL2/3 for HLA-C1 in haplotype B, based on their bead-based assay (Hilton et al. 2015). However, our results did not distinctly demonstrate such trends, although we observed the highest avidity for C2 in KIR2DL1*003 and low avidity of KIR2DL3*002 for C1, both located in haplotype A. A more comprehensive understanding could have been achieved by expanding the range of HLA-C/KIR2D combinations assessed, potentially accounting for differences present in different clades. The disparities in observations may also stem from methodological differences; our utilization of a cell-based assay contrasts with bead-based assays used in previous studies. As HLA-C is most likely peptide-loaded, differences in comparisons to different HLA-C alleles coated on beads might have contributed to variations observed. This is supported by a study by Sim et al., which utilized HLA-C molecules with identical sequences but differing at KIR-specificity-determining positions. When examining the HLA-C2 molecule, they found that the vast majority of loaded peptides still supported KIR2DL1 binding. In contrast, for HLA-C1, only a subset of loaded peptides facilitated KIR2DL2/2DL3 binding (Sim et al. 2017).

In the context of HIV-1 infection, the significance of binding affinities between KIR and HLA-I molecules has been consistently observed. High binding affinities of KIR and HLA-I during NK cell development are crucial for driving NK cell education (Brodin et al. 2009; Boudreau et al. 2016). Depending on the level of education, NK cells can respond more rapidly and robustly to subtle changes in HLA-I expression on target cells. Studies conducted by Kiani et al.

demonstrated that educated KIR2D+ NK cells respond in an affinity-based manner. They assessed NK cell response by measuring degranulation, IFN-γ, and CCL4 production after exposure to autologous HIV-1-infected CD4 cells using the HLA-C downmodulating HIV strain JR-CSF. Consistent with our observed binding avidities, they observed that single-educated KIR2DL1 cells exhibited the highest anti-HIV activity, while KIR2DL2 single-educated cells produced more CCL4 compared to single-educated KIR2DL3 NK cells (Kiani et al. 2019). The significance of education in HIV is further outlined by the findings of Jennes et al., who suggested that serodiscordant couples were more likely to comprise an uninfected recipient partner with an *KIR/HLA* combination educating NK cells, such as KIR2DL1+HLA-C2, paired with an HIV+ partner homozygous for C1, compared to seroconcordant couples (Jennes et al. 2013). Furthermore, in a Japanese cohort of untreated HIV-infected individuals, it was reported that individuals expressing KIR2DL2 with *HLA-C* alleles *12:02* and *14:03*, presenting the C1 motif, suppressed viral replication compared to controls. However, pulsing with Gag-peptides displayed reduced stability of the HLA-C:Gag-peptide complex, leading to higher responses from KIR2DL2 NK cells (Lin et al. 2016). Several reports have demonstrated that HIV peptides not only cause lower stability of HLA-I molecules but also affect the binding affinity to KIR receptors. Fadda et al. used a library of overlapping HIV-1 p24 peptides, pulsed T2 cells expressing HLA-C*0102, and identified 11 peptides that stabilized the HLA-C:peptide complex. Intriguingly, one of these complexes was able to inhibit KIR2DL2-educated NK cells, providing protection against NK cell-mediated lysis (Fadda et al. 2012). A similar approach was undertaken using tapasin-deficient 721.220 cells expressing HLA-C*03:04. Subsequent pulsing with HIV-1 p24 Gag-derived peptides identified numerous novel Gag peptides that stabilized HLA-C*03:04. Additionally, three of these peptides, when presented on HLA-C, were able to bind and inhibit KIR2DL2+ NK cells. Notably, naturally occurring variants of Gag peptides were also found to engage KIR2DL2+ NK cells and enhance binding, thereby inhibiting NK cell function (van Teijlingen et al. 2014). This underscores that HIV modulates binding affinities between KIR2DL and HLA-C to modulate the host immune response. This notion is supported by p24 Gag mutations found in an untreated HIV-1 cohort in South Africa with a distinctive *KIR2DL3/HLA-C*03:04* genotype (Hölzemer et al. 2015). Unlike previous peptide-pulsing experiments, these mutations had undergone immune pressure selection within the host. The mutated Gag epitope, presented on HLA-C, exhibited stronger engagement of KIR2DL3+ NK cells, resulting in decreased NK cell degranulation after exposure to target cells presenting the variant epitope. However, studies have shown that only 0.2% of peptides presented by HIV-1-infected cells are derived from HIV. Moreover, the most abundant HLA-C-presented peptide by HIV-1-infected cells was unable to engage KIR2DL3 and thus could not inhibit NK cell function (Ziegler et al. 2020). These studies provided the first insights into how the HLA-C peptidome in HIV-1 infection influences KIR2DL binding, but

several limitations must be addressed in future research. First, most investigations were conducted by peptide pulsing of cells which represents an artificial method of investigating peptides presented by HLA-C molecules. Second, prior studies have primarly focused on p24 Gag-derived peptides (Hölzemer et al. 2015; Ziegler et al. 2020; van Teijlingen et al. 2014), potentially overlooking other HIV-1 peptides that may contribute to the HLA-C-presented repertoire (Chikata et al. 2019). Additionally, experiments have relied on the NL4-3 HIV-1 strain, which does not downregulate HLA-C. Consequently, the selective pressure for HIV-1 to present peptides that facilitate enhanced KIR2DL binding is likely lower, given that HLA-C molecules remain abundant on the cell surface. Furthermore, previous studies have examined HIV-1-infected cells in the absence of NK cell-mediated immune pressure. In contrast, our study provides evidence that NK cell pressure selects for specific HIV-1 accessory protein sequence variations, as demonstrated by the association between high KIR2DL/HLA-C binding affinities and Vpu amino acid sequences that facilitate HLA-C downregulation. These findings align with those of Hölzemer et al., supporting the notion that KIR-HLA binding affinity contributes to the host immune pressure, driving the selection of viral protein variants (Hölzemer et al. 2015). Lastly, studies have largely focused on HIV-1-derived peptides presented on HLA-C, despite the fact that HIV-1 infection itself alters the presentation of host-derived peptides. So far, this has only been investigated using NL4-3, where infection led to the loss of KIR2DL3 binding to HLA-C1 (Ziegler et al. 2020). Future research should address these gaps to fully understand the interplay between HIV-1, HLA-C presentation, and NK cell recognition. Apart from HIV-1, other viral infections also modulate KIR2D/HLA-C binding to evade NK cell-mediated immune response (Charoudeh et al. 2013; Lunemann et al. 2016). These collective findings provide insights that host-predetermined KIR2DL/HLA-C binding affinities are manipulated by HIV-1 to evade NK cell-mediated immune pressure.

In the past decade, various research groups have elucidated the role of the HIV-induced downregulation of HLA-C, primarily mediated by the viral accessory protein Vpu (Apps et al. 2016; Ende et al. 2018; Körner et al. 2017). Notably, studies have highlighted the significant variability of the Vpu region within the HIV-1 genome (Yusim et al. 2002; Verma et al. 2013). This variability is believed to be influenced by host immune pressure, although the precise selection pressures favoring certain Vpu variants remain unclear (Pickering et al. 2014; Bachtel et al. 2018). Investigations into the impact of cytotoxic T lymphocytes (CTLs) have shown that mutations in Vpu sequences tend to accumulate within epitopes predicted to be presented on HLA-I molecules, suggesting an active evasion of the immune response (Pickering et al. 2014). As another mechanism effecting Vpu polymorphism, Bachtel et al. explored the association between Vpu-mediated HLA-C downregulation and initial high HLA-C expression based on the individual's underlying genotype, proposing that Vpu downregulates highly expressed HLA-C alleles to evade HLA-C restricted CTL responses (Bachtel et al. 2018). This hypothesis is

supported by studies identifying HLA-C-restricted CTLs in untreated chronically HIV-1-infected individuals, demonstrating their ability to suppress viral replication and recognize HIV-derived epitopes *in vitro* and high HLA-C expression levels correlate with slower progression to AIDS (Apps et al. 2013; Honda et al. 2011; Honeyborne et al. 2010; Makadzange et al. 2010; Thomas et al. 2009). The significance of HLA-C expression levels was also evidently shown by a Genome Wide Association Study (GWAS), which identified a SNP in the 5' region of HLA-C that is associated with higher expression and lower viral load (Fellay et al. 2007). However, Bachtel et al. also investigated the impact of NK cell immune pressure on Vpu variants, observing no differences in the frequency of Vpu clones that extensively downregulate HLA-C when stratifying *HLA-C* alleles into *C1* and *C2* groups. Notably, this study did not consider NK cell education processes or the varying ability of KIRs to bind their cognate HLA-C molecules, although prior investigations have shown that downregulation of HLA-C on *in vitro* HIV-infected CD4 cells correlated with susceptibility to NK cell suppression of virus release (Ende et al. 2018). In contrast, our investigation accounted for these differences, focusing on the impact of KIR2D/HLA-C genotype on Vpu sequence polymorphism. Yet we had no information of HLA-C expression in our cohort prior to HIV-1 infection, preventing us from considering the potential influence of initial HLA-C expression on Vpu polymorphism. Recent research has identified specific amino acid residues in the transmembrane and extracellular domain of Vpu associated with HLA-C downregulation (Bachtel et al. 2018). We examined variations in these amino acid positions in correlation with our established KIR2D/HLA-C binding affinities. To accomplish this, we analyzed 122 samples obtained from untreated HIV+ individuals. Our investigation involved assessing Vpu variants isolated from both plasma and PBMCs to account for both replicative and integrated variants. Interestingly, we observed that Vpu variants derived from plasma samples exhibited an enhanced ability to downregulate HLA-C, which coincided with HLA-C/KIR2DL combinations showing stronger binding affinities. To contextualize our findings regarding the downregulation of highly expressing HLA-C alleles, we propose a mechanism by which HIV-1 calibrates HLA-C expression to evade both CTL and NK cell responses. Downregulation of highly expressing HLA-C molecules reduces CTL recognition while maintaining sufficient expression to inhibit NK cells. Additionally, reducing HLA-C with high binding affinities to KIR2D molecules, while still conserving a certain baseline of HLA-C expression, prevents NK cell activation. Whereas KIR2D/HLA-C binding partners with lower binding intensities may sense subtle changes in HLA-C expression and become activated. In conclusion, Vpu modulates HLA-C expression to evade both NK cell and CTL recognition, suggesting a complex interplay between HIV-1, host genetics, and the immune response. A comprehensive approach, including testing of individuals for HLA-C expression prior HIV-infection and information regarding HLA-C/KIR2D binding affinities, is warranted. Moreover, expanding the tested cohort is essential given the vast diversity in HLA-C/KIR2D combinations.

Despite the complexity introduced by NK cells, our study underscores the importance of considering KIR2DL+ NK cell-mediated immune pressure in understanding the impact of Vpu polymorphism on immune evasion.

The expansion of NK cells in response to viral infections is a well-established phenomenon, leading to profound alterations in NK cell subset composition (Mujal et al. 2021). Among these alterations, the expansion of NKG2C+ NK cells in response to CMV infection stands out, characterized by heightened proliferative activity and enhanced antibody-dependent cellular cytotoxicity (Hammer et al. 2018; Sun und Lanier 2009; Rölle et al. 2018). In the context of HIV-1 infection, significant changes in the NK cell receptor profile have been documented, independent of individual genotypes but also in a KIR-dependent manner (Pelak et al. 2011; Mavilio et al. 2003; Maria et al. 2003; Ahmad et al. 2001b; Alter et al. 2007; Alter et al. 2009). In our study, we aimed to comprehensively investigate an array of NK cell receptors in untreated HIV-infected individuals compared to an HIV- control group. We focused our analysis on KIR2D+ NK subsets, known to interact with HLA-C, and included well-established NK cell subset markers such as NKG2A, NKG2C, CD57, and CD8 to provide a comprehensive phenotypic characterization of NK cells in the context of chronic HIV-1 infection. Consistent with previous reports, we observed a lower frequency of CD8+ NK cells in untreated HIV+ compared to HIV- individuals. In a longitudinal retrospective study involving 117 untreated HIV-infected subjects, the decline of CD8+ NK cells correlated with HIV viral loads and inversely correlated with CD4 T cell counts. Notably, the CD8+ NK cells in this untreated cohort exhibited elevated CD57 expression, indicative of a mature subset (Ahmad et al. 2014). Subsequent studies have shown a decline in CD57 expression in NK cell subsets during chronic HIV infection (Hong et al. 2010). Generally, NK cells diversify after viral challenges and CD57 expression increases by cytokine exposure, suggested to be caused by encountered pathogens (Lopez-Vergès et al. 2010; Strauss-Albee et al. 2014). Notably, in an African women cohort with a high HIV-1 exposure risk, a predisposed high NK cell diversity has been shown to have an increased risk of HIV-1 infection (Strauss-Albee et al. 2015). However, the interpretation of elevated CD57 expression in HIV infection is confounded by its often observed co-expression with NKG2C-expanded NK cell subsets, which are characteristic for CMV infection (Heath et al. 2016). Indeed, alterations in NKG2C levels are closely associated with concurrent CMV infection rather than solely HIV-1 infection yet are still enhanced when comparing HIV+ and HIV- individuals who are seropositive for CMV (Gumá et al. 2006; Hearps et al. 2017; Mela und Goodier 2007). CMV is known to induce NKG2C-expressing memory-like NK cells with enhanced antibody-dependent cellular cytotoxicity and cytokine secretion in response to CD16 engagement (Hwang et al. 2012; Lee et al. 2015). Incorporating CMV serological status into multivariate regression analyses has been shown to nullify the observed positive correlation between elevated NKG2C+ NK cell levels and HIV-1 infection, suggesting

that the expansion of NKG2C+ NK cells during HIV-1 infection is primarily driven by CMV co-infection (Gumá et al. 2006). Remarkably, the prevalence of NKG2C+CD57+ NK cells remain exceedingly low or undetectable in individuals who are seronegative for CMV, regardless of their HIV-1 status (Heath et al. 2016). Interestingly, individuals harboring a deletion in the NKG2C gene were identified among people living with HIV compared to HIV-exposed seronegative individuals, suggesting a potential role for NKG2C in antiviral defense mechanisms (Alsulami et al. 2021). However, it is noteworthy that this study did not specify whether prior CMV exposure occurred in individuals with the NKG2C deletion, as CMV has been shown to drive expansion of memory-like NK subsets even in individuals lacking the NKG2C gene (Comeau et al. 2019; Della Chiesa et al. 2015). Although elevated levels of NKG2C+ NK cells has been documented in response to hantavirus infection or chronic hepatitis, this phenomenon still predominantly manifests in individuals who are seropositive for CMV (Béziat et al. 2012; Björkström et al. 2011). Nevertheless, Gondois-Rey and colleagues reported that CMV-primed NK cell subsets expand in response to HIV-1 infection, as they observed higher frequencies of NKG2C+ NK cells in acutely and early HIV-1-infected individuals without ongoing CMV reactivation compared to healthy donors (Gondois-Rey et al. 2017). Yet, the study did not provide information on the CMV status of the healthy donors, which would have been necessary to support this conclusion. Since the CMV status of study subjects in our HIV+ cohort was not available, we were unable to stratify donors according to their CMV serological status. Nevertheless, our findings align with previous results showing an inversion of the NKG2A/NKG2C ratio in advanced stages of disease in CMV+ HIV-infected individuals, characterized by a downmodulation of the inhibitory receptor NKG2A and a simultaneous increase in NKG2C-expressing NK cells (Brunetta et al. 2010). Both NKG2A and NKG2C engage HLA-E as their cognate binding partner, and several studies have characterized HLA-E expression on HIV-infected cells (Nattermann et al. 2005; Bonaparte und Barker 2004; van Stigt Thans et al. 2019). Manipulating the interplay between NKG2C, as an activating receptor, and NKG2A with their binding partner HLA-E by HIV is critical, as NKG2A+ NK cells have been shown to respond effectively to HIV-infected cells (Davis et al. 2016; Lisovsky et al. 2015; Ramsuran et al. 2018).

Numerous investigations have delineated genotype-dependent expansions of KIR+ NK cells in untreated HIV-1-infected individuals. Within our cohort, a significant reduction in the frequency of KIR2DS4+ NK cells was evident, while no significant differences in KIR expression on bulk NK cells were observed compared to HIV- controls. Prior studies have elucidated KIR2DS4 expression in healthy individuals exposed to HIV and have compared its expression with recently HIV-infected subjects. Interestingly, these studies demonstrated lower expression levels in healthy HIV-exposed individuals compared to HIV+ individuals, indicating reduced resistance against HIV carrying the full-length transcript of KIR2DS4 (Middleton et al.

2007; Jackson et al. 2017). Moreover, KIR2DS4 has been associated with adverse outcomes in HIV infection, such as higher viral loads, lower CD4 counts, and HIV transmission in serodiscordant couples (Merino et al. 2011; Merino et al. 2014). Consistent with these findings, Olvera et al. identified elevated viral loads in KIR2DS4-positive individuals concurrently expressing its putative ligand, HLA-C*04:01 (Olvera et al. 2015). Less is known about KIR2DS1 frequencies in HIV cohorts. One study observed lower frequencies of KIR2DS1+ or KIR2DL1+ cells among individuals with higher viral copy numbers, albeit using an antibody with cross-reactivity between KIR2DS1 and KIR2DL1, yielding indistinct results (Hong et al. 2010). In contrast, our study employed a combination of antibodies distinguishing between KIR2DL1 and KIR2DS1, revealing no significant differences in KIR2DL1 or KIR2DS1 expression on bulk NK cells compared to HIV- controls. However, stratification based on *HLA-C1* and *HLA-C2* alleles unveiled an expansion of KIR2DL1+ NK cells within the HIV+ cohort among individuals carrying homozygous *C2/C2*, a phenomenon not observed in the HIV- group. These results are consistent with findings by Körner et al., who also observed similar trends for KIR2DL2/3-positive NK cells (Körner et al. 2014). To link binding strength to subset expansion, we correlated obtained binding affinities between KIR2DL/HLA-C and KIR2DL frequencies in HIV- and HIV+ individuals. We found a significant positive correlation between the strength of KIR2DL1 engagement with the C2 motif and the expansion of KIR2DL1+ NK cells, not observed in HIV- individuals. However, this correlation was absent for KIR2DL2/3+ NK cells and the C1 motif. Notably, tested binding avidities of KIR2DL2/3 with C1 were overall weaker, suggesting that HIV+ individuals carrying these genotypes, tested in our binding avidity assay, may not result in an expansion of low-affinity binding KIR2DL2/3 NK cells. Epidemiological studies have reported associations between *KIR3DL1/HLA-B* genotypes and disease progression in HIV-1 infection (Martin et al. 2002; Martin et al. 2007). Several of these studies focus on the relationship between inhibitory KIR3DL1, activating receptor KIR3DS1, and HLA-B in the context of infection resilience or disease progression (Tallon et al. 2014; Ravet et al. 2007; Martin et al. 2002; Jiang et al. 2013; Habegger de Sorrentino et al. 2013). For instance, a study in a Vietnamese cohort of injection drug users found that HIV-exposed healthy individuals had higher ratios of KIR3DS1:KIR3DL1 transcripts compared to uninfected individuals and individuals living with HIV (Ravet et al. 2007). In our study, we observed no differences in KIR3DL1 expression between HIV- and HIV+ individuals. However, further assessment to correlate KIR3DL1/HLA-B combinations with viral loads and CD4 counts was not performed. Overall, while most KIR gene association studies have focused on the presence or absence of certain *KIR3D* alleles, few have considered the impact of strong binders for KIR3DL1, such as HLA-B*57. However, the polymorphic landscape of KIR2DL and its cognate binding partner HLA-C has not been adequately addressed. Each combination of KIR2DL/HLA-C exhibits differential binding affinities and consequently impacts NK cell

function. This study lays the groundwork for accounting various KIR2DL/HLA-C matches and their KIR2DL subset modulation by HIV-1 infection. Additionally, our study highlights the challenges in predicting disease course and HIV infection susceptibility, given the various factors in NK cell biology contributing to HIV control.

In summary, this study aimed to delve into the complex interplay between KIR2DL/HLA-C combinations and the consequent effects on NK cell anti-HIV-1 activity, alongside the alterations induced by HIV-1 in the NK cell receptor repertoire. Furthermore, Vpu sequence polymorphisms were analyzed and linked to HLA-C/KIR2D binding affinities as a potential HIV-1 immune escape mechanism. Our findings suggest a mechanism by which HIV-1 modulates HLA-C expression to evade NK cell responses. Downregulation of highly expressing HLA-C molecules enables evasion of CTL recognition while maintaining sufficient expression to inhibit NK cells. Additionally, reducing HLA-C molecules with high binding affinities to KIR2D molecules, while retaining a baseline of HLA-C expression, prevents NK cell activation. Conversely, KIR2D/HLA-C binding partners with lower binding intensities may detect subtle changes in HLA-C expression and become activated. To validate this hypothesis, further investigations are necessary. Consequently, future steps should focus on exploring the interplay of binding avidities and the elimination of autologous *in vitro* infected CD4 T cells using virus strains with reduced capability to downmodulate HLA-C. According to our hypothesis, autologous HIV-1-infected CD4 T cells maintaining adequate HLA-C expression levels may be more vulnerable to KIR2D-educated NK cells with lower binding affinities, as high HLA-C expression can retain NK cell activity with high HLA-C binding affinities. Given that the strength of KIR2DL+ NK cell education correlates directly with their response magnitude in the absence of the cognate HLA-C molecule, employing HIV-1 strains with high downmodulation ability of HLA-C would result in infected CD4 T cell killing primarily by KIR2D-educated NK cells with high affinity for HLA-C. This observation would raise the intriguing possibility of employing monoclonal antibodies in the early stages of HIV-1 infection to inhibit KIR2D and HLA-C interactions. Such an approach has already demonstrated clinical relevance in various settings and could potentially bolster NK cell-mediated clearance of infected cells. For example, the anti-KIR monoclonal antibody IPH2101, formerly 1–7F9, exhibits high affinity binding to KIR2D receptors and enhances NK cell-mediated elimination of autologous HLA-C-expressing leukemic cells *in vitro* in a dose-dependent manner (Romagné et al. 2009). Given the variability in viral-mediated downregulation of cell surface HLA-C impacting NK cell antiviral activity, IPH2101 may be particularly relevant in HIV-1-infected individuals to completely abrogate KIR2D/HLA-C binding, effectively mimicking a KIR-HLA mismatch and improving the elimination of infected cells, potentially preventing the establishment of viral reservoirs. Additionally, considering the confounding effects, evaluating the plasma of untreated HIV+ individuals concerning their serological CMV-state is essential. CMV-induced adaptive NK

cells, characterized by high KIR expression levels and altered epigenetic composition that lead to higher responsiveness, may skew the effects of KIR2D+ NK cells, particularly in assessing their activity based on binding affinities (Schlums et al. 2015; Lee et al. 2015; Béziat et al. 2013).

The second study investigated TRAIL's involvement in HIV-1 infection, revealing its multifaceted functionality. TRAIL, expressed by various immune cells (Sag et al. 2019), emerged as an effective candidate for recognizing HIV-1-infected cells during comprehensive immunological screening. Subsequent efforts focused on delineating TRAIL expression in specific NK cell subsets. TRAIL expression can be induced by cytokines such as IFN-γ, IL-2, and IL-15, leading to elevated levels of TRAIL in NK cells supplemented with these cytokines (Takeda et al. 2001; Kayagaki et al. 1999b). Consequently, we observed preferential TRAIL expression on $CD56^{bright}$ and NKG2A+ NK cells, aligning with previous studies indicating that $CD56^{bright}$ cells are responsive to IL-15 stimulation. (Carson et al. 1994; Nielsen et al. 2012; Wagner et al. 2017) This enhanced TRAIL expression on $CD56^{bright}$ NKG2A+ NK cells may contribute to their potent antiviral response, as documented by their augmented response against HIV-1-infected CD4 T cells observed in other studies (Lisovsky et al. 2015).

Furthermore, stratification of NK cell subsets into CD107a- and CD107a+ NK cells revealed that TRAIL expression was enhanced in degranulated NK cells across all assessed subsets, suggesting its involvement in triggering NK cells to release cytotoxic granules. To confirm TRAIL's role in degranulation, NK cells were exposed to an HLA-class I deficient cell line 221 in the presence of an αTRAIL blocking-antibody. This resulted in reduced degranulation of bulk NK cells compared to the isotype control. Previous studies have reported decreased CD107a expression following co-culture of primary hepatic stellate cells (HSC) and the hepatic stellate cell line-LX2 with IL-18 and poly I:C-stimulated NK cells in presence of an αTRAIL antibody (Li et al. 2019). Employing soluble TRAIL instead of αTRAIL antibody to block death receptors on target cells similarly reduced NK cell degranulation. The authors observed that blockade of TRAIL inhibited the interaction between NK cells and LX2 cells. Our results, however, did not show a significant contribution of TRAIL in conjugate formation between NK cells and 721.221 cells. Additionally, blocking DR4 and DR5 in the underlying study, the receptors for TRAIL, also led to decreased degranulation, albeit to a lesser extent than with the αTRAIL antibody. Decoy receptors, which are not neutralized by DR4 and DR5 antibodies, can still interact with TRAIL, providing an explanation for our observations. The finding that OPG and DcR1 can induce NK cell degranulation when coated on a plate supports this notion. Interestingly, OPG, functioning as a soluble factor, as well as decoy receptors, lack a death domain and therefore cannot initiate apoptosis (Degli-Esposti et al. 1997a; Degli-Esposti et al. 1997b; Emery et al. 1998).

Various cancer cell lines exploit this characteristic of OPG, where its overexpression protects against TRAIL-induced cell death (Zauli et al. 2009; Sandra et al. 2006; Neville-Webbe et al. 2004; Holen et al. 2002). Decoy receptors are frequently overexpressed in tumors, serving to dampen TRAIL-induced apoptosis (Bai et al. 2000; Pitti et al. 1998). Mérino et al. provided insights into the mechanism by which decoy receptors suppress TRAIL-mediated cytotoxicity (Mérino et al. 2006). Typically, TNF receptors form homotrimeric complexes; however, the preligand assembly domains in DR5 and DcR2 permit mixed complex formation, thereby inhibiting initiator caspase activation. Conversely, DcR1 localizes in raft microdomains and competes with TRAIL receptors to inhibit TRAIL-induced apoptosis (Mérino et al. 2006; Clancy et al. 2005). The discovery that DcR1 and OPG induce degranulation adds a new role for these receptors as they potentially both protecting tumor cells from TRAIL-induced apoptosis and enhancing TRAIL-mediated anti-tumor activity of NK cells. This insight offers novel perspectives on the functions of DcR1 and OPG in NK-cell-mediated tumor surveillance. While TRAIL is well known for its role in tumor biology, its involvement in viral infections is increasingly recognized.

The specific role of TRAIL in the NK cell response against HIV-1 was investigated by co-culturing bulk NK cells with autologous HIV-1-infected CD4 T cells in the presence and absence of a αTRAIL blocking antibody. Addition of the αTRAIL antibody led to a decrease in NK cell degranulation. TRAIL's significant involvement in controlling viral diseases has been demonstrated in various contexts, including the *in vivo* replication of encephalomyocarditis virus in mice and the *in vitro* elimination of hepatitis C virus (HCV)-replicating hepatoma cells through TRAIL-mediated cytotoxicity (Stegmann et al. 2010; Sato et al. 2001). CMV has also been demonstrated to downregulate TRAIL receptor expression on infected cells as a mechanism to evade NK cell-mediated elimination, underscoring the critical role of TRAIL-mediated cytotoxicity in pathogen clearance (Verma et al. 2014). In HIV-1 infection, although limited data exist on the involvement of TRAIL-mediated cytotoxicity of NK cells, a study by Lum et al. reported increased TRAIL-mediated cytotoxicity against the cancer cell line K562 by IL-15-stimulated NK cells from HIV-1-infected patients (Lum et al. 2004). There is substantial evidence supporting TRAIL's involvement in both the pathogenesis of HIV-1 and its control, which reinforces our initial finding of increased TRAIL expression in degranulating NK cells upon exposure to autologous HIV-1-infected CD4 T cells (Gougeon und Herbeuval 2012). TRAIL is a component of the immediate immune response, with elevated plasma levels detectable as early as 7 days before the peak of plasma viral load (Gasper-Smith et al. 2008). This aligns with findings by Herbeuval et al., who observed elevated serum levels of TRAIL in HIV-1-infected patients compared to uninfected healthy individuals, where higher TRAIL concentrations are associated with higher viral loads (Herbeuval et al. 2005b). Patients undergoing ART exhibit reduced plasma TRAIL levels, correlating with viral load reduction, and

poor CD4+ T cell recovery in response to ART has been linked to increased TRAIL receptor expression (Herbeuval et al. 2005a; Hansjee et al. 2004). Upregulation of TRAIL receptors on CD4 T cells has been detected in numerous reports, rendering them more susceptible to TRAIL-mediated cytotoxicity (Herbeuval et al. 2009; Herbeuval et al. 2005b). This observation is consistent with the upregulation of DR4 and DR5 in our *in vitro* HIV-1 infection of CD4 T cells, and previous studies demonstrating that treatment of uninfected Jurkat T cells, as well as primary T cells, with gp120 results in increased TRAIL death receptor expression and acquired sensitivity to TRAIL-mediated cell death (Lum et al. 2005). The significance of the TRAIL-TRAIL receptor axis in viral infection regulation is underscored by the upregulation of TRAIL by various immune cells (Herbeuval et al. 2005c; Herbeuval et al. 2005b; Hardy et al. 2007; Stary et al. 2009).

Our plate coating experiments, incorporating a TRAIL blocking condition, unequivocally demonstrate that TRAIL engagement triggers degranulation and IFN-γ production. However, the absence of a cytoplasmic domain capable of transmitting a signal raises questions about the intracellular signaling pathways that lead to degranulation and IFN-γ release (Cha et al. 1999; Ehrlich et al. 2003). Early studies have shown that members of the TNF-family can induce signaling upon engagement, a phenomenon known as reverse signaling. For example, enhanced proliferation has been demonstrated following FasL binding in CD8+ T cells, which share cytotoxic features with NK cells (Suzuki und Fink 2000, 1998). Shortly thereafter, reports by Chou et al. in 2001 documented similar findings for TRAIL. They used plate-bound DR4-Fc fusion proteins to induce TRAIL-mediated signaling, resulting in enhanced proliferation of mouse T cells and increased IFN-γ production (Chou et al. 2001). However, TRAIL engagement alone was insufficient to induce proliferation or cytokine production; rather, it acted as a co-stimulatory molecule in combination with T cell receptor engagement. The same group observed similar results with human T cells, showing that TRAIL engagement selectively activated human CD4, rather than CD8, T cells (Tsai et al. 2004). Reports on TRAIL signaling in NK cells are scarce. Data indicate that TRAIL-deficient NK cells exhibit reduced granzyme B expression, associated with impaired NK cell-mediated cytotoxicity (Cardoso Alves et al. 2020). However, when TRAIL-deficient NK cells were exposed to YAC-1 cells, they displayed similar CD107a+ expression compared to wildtype NK cells. Additionally, they reported higher expression levels of IFN-γ by TRAIL-deficient NK cells. Gene expression analysis revealed differences in several pathways related to inflammation, cytokine production, or signaling between TRAIL-deficient and wildtype NK cells upon LCMV infection. These results suggest that TRAIL engagement can induce reverse signaling and potentially affect intracellular signaling pathways. They conclude that TRAIL+ NK cells contribute to the restriction of specific CD8 T cell responses in their LCMV infection model in mice. Regulatory functions of TRAIL+ NK cells are also observed in MCMV infection, where TRAIL+ NK cells eliminate activated CD4

T cells in salivary glands (Schuster et al. 2014). Overall, studies observing reverse signaling of TRAIL are rare. Reports in humans have only described TRAIL's role as a potential co-stimulatory molecule, particularly in CD4 T cells. Other studies have derived their findings regarding TRAIL's reverse signaling from mouse models, where they also found no difference in degranulation of TRAIL-deficient mice, contradicting findings of lower degranulation of human NK cells subjected to hepatic stellate cells after blocking TRAIL (Cardoso Alves et al. 2020; Li et al. 2019). This disparity could suggest different mechanisms in mice and humans regarding TRAIL's ability to induce reverse signaling.

We sought out to identify signaling molecules in NK cells phosphorylated upon TRAIL engagement. Since TRAIL lacks a cytoplasmic domain capable of transmitting a signal, elucidating the pathway activated by TRAIL presents a challenge. Many activating NK cell receptors feature a positively charged amino acid (lysine or arginine) in the transmembrane domain, enabling association with adapter molecules like DAP12, DAP10, or CD3ζ. Recruitment of these adapters triggers downstream activation of Syk, PLC-γ2, and Akt, crucial for IFN production and degranulation (Upshaw et al. 2005; Sutherland et al. 2002; Spaggiari et al. 2001; Jiang et al. 2002) . However, TRAIL lacks a positively charged amino acid in its transmembrane domain, making targeted recruitment of adapter molecules improbable. Syk, PLC-γ2, and Akt can independently activate pathways leading to cytokine production, calcium influx, and degranulation (Meza Guzman et al. 2020). Pathway activation includes also members of the MAP kinases, such as p38. Activation of MAP kinase p38, initiated by FcεRIg or CD3ζ activation in NK cells, regulates cytotoxicity (Lanier et al. 1991; Trotta et al. 2000). An early study showed p38 phosphorylation and IFN-γ release upon TRAIL co-stimulation, while the p38 inhibitor SB 203580 suppresses IFN-γ secretion (Tsai et al. 2004). This suggests that TRAIL engagement may hijack nearby adapter molecules capable of transmitting an activation signal. This phenomenon has been described for CD59. CD59 can serve as a co-receptor for activating receptor NKp46, bearing CD3ζ adapter molecules, promoting signaling and cytotoxicity despite lacking its own signaling domain (Marcenaro et al. 2003). However, our evaluation of the phosphorylation of signaling molecules revealed elevated baseline phosphorylation levels, possibly attributed to extended exposure to IL-2 and IL-15, with similar findings observed in murine NK cells (Luu et al. 2021). Further investigation into the role of these molecules in TRAIL signaling yielded inconclusive results, as our experimental setup aimed to measure dephosphorylation after signaling cascade completion.

Another partially explored mechanism is TRAIL's association with lipid rafts, which harbor signaling molecules and play a role in compartmentalizing signaling pathways (Mollinedo und Gajate 2020; Foster et al. 2003). Besides signaling molecules, varieties of molecules are aggregated in these lipid rafts. As part of the TNF protein family, FasL is described to be

constitutively localized in rafts (Cahuzac et al. 2006). Another report linked TRAIL engagement to lipid raft-induced intracellular signaling. They demonstrated that TRAIL stimulation via DR4-Fc induced phosphorylation of kinases Lck and ZAP70, resulting in activation of the downstream NF-kB pathway (Huang et al. 2011). However, TRAIL ligation alone did not induce T cell proliferation. In contrast, co-stimulation of CD28 and TRAIL led to lipid raft assembly, which is essential for effective T cell receptor signal transduction (Huang et al. 2011; Montixi et al. 1998; Xavier et al. 1998). The assembled liquid raft leads to recruitment of Lck, whereas TRAIL ligation or CD28 ligation alone was insufficient to induce lipid raft recruitment of Lck. Notably, signaling molecules Lck and ZAP70 play a vital role in NK cell activation. Inhibition or knockdown of Lck resulted in significant reductions in NKG2D- and CD137-mediated cytotoxicity and cytokine production in NK cells (Rajasekaran et al. 2013), whereas phosphorylation of ITAMs results in ZAP70 activation in an Lck dependent manner (Lanier et al. 1998b; Ting et al. 1995; Vivier et al. 1993). Our results, alongside published reports on NK cell activation after TRAIL stimulation, suggest the need for further inquiry and exploration, as a definitive mechanism or activated pathway remains uncertain.

In conclusion, our study identified TRAIL as a crucial effector molecule in the response to HIV-1-infected cells. While TRAILs ability to induce receptor mediated cytotoxicity is well-established, we uncovered a novel aspect by demonstrating its direct role in triggering NK cell degranulation and IFN-γ release. This finding proposes a reverse signaling mechanism for TRAIL in NK cells, expanding beyond its known function in receptor-mediated cytotoxicity. A comprehensive screening of the phospho-signaling network in NK cells after TRAIL engagement is a crucial next step. One method, Kinase-interacting substrate screening (KISS) analysis, could be employed for this purpose (Amano et al. 2016). In this approach, NK cells would be stimulated with TRAIL, and proteins would be extracted for analysis. By incubating extracted proteins with bait substrates coupled to beads, kinase-substrate complexes can be formed and undergo *in vitro* phosphorylation. Subsequent digestion and mass spectrometry analysis would identify essential kinases involved in TRAIL-induced signal transduction, shedding light on the underlying signaling pathways. Another approach could involve investigating the possibility of TRAIL hijacking domains of proxy activating receptors. In this method, NK cells stimulated with αTRAIL would be lysed, and cell lysates subjected to TRAIL immunoprecipitation. Analysis of phosphorylated adapter molecules by Western blot could reveal potential candidates involved in TRAIL-mediated signaling. Additionally, systematic screening for phosphorylated immunoreceptors after TRAIL stimulation using available kits could provide valuable insights. Once potential candidates are identified based on the phosphorylation of immunoreceptors, they would be employed to immunoprecipitate cell

lysates post-TRAIL stimulation. Subsequent Western blot analysis aims to validate the phosphorylation of signaling molecules associated with activating receptors. Corresponding findings between TRAIL-precipitated lysates and immunoreceptors-precipitated lysates would provide supportive evidence for this proposed mechanism. Furthermore, the potential hijacking phenomenon could be further investigated through immunofluorescence assays to assess the co-localization of TRAIL and the activating receptor. While TRAIL's potential in tumor therapy has been explored with mixed results, our study further underscores its potential as a target for NK cell-based immunotherapeutic approaches in antiviral therapies (Snajdauf et al. 2021). However, to fully elucidate its mode of action, further investigation is warranted.

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5 Declarations of generative AI and AI-assisted technologies in the writing process

During the preparation of this thesis, the AI tool ChatGPT-4 was used exclusively to assist with language refinement and clarity. No original content or text was generated by the AI. Rather, existing paragraphs written by the author were input into the tool for revision and polishing. All content was reviewed and edited afterward, and I take full responsibility for the final version of the text.

6 Affidavit

I hereby declare and affirm that this doctoral dissertation is my own work and that I have not used any aids and sources other than those indicated.

If electronic resources based on generative artificial intelligence (gAI) were used in the course of writing this dissertation, I confirm that my own work was the main and value-adding contribution and that complete documentation of all resources used is available in accordance with good scientific practice. I am responsible for any erroneous or distorted content, incorrect references, violations of data protection and copyright law or plagiarism that may have been generated by the gAI.

Hamburg, 09.05

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7 Appendix

Article

SOURCE DATA | TRANSPARENT PROCESS | OPEN ACCESS

EMBO reports

Engagement of TRAIL triggers degranulation and IFNγ production in human natural killer cells

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Abstract

NK cells utilize a large array of receptors to screen their surroundings for aberrant or virus-infected cells. Given the vast diversity of receptors expressed on NK cells we seek to identify receptors involved in the recognition of HIV-1-infected cells. By combining an unbiased large-scale screening approach with a functional assay, we identify TRAIL to be associated with NK cell degranulation against HIV-1-infected target cells. Further investigating the underlying mechanisms, we demonstrate that TRAIL is able to elicit multiple effector functions in human NK cells independent of receptor-mediated induction of apoptosis. Direct engagement of TRAIL not only results in degranulation but also IFNγ production. Moreover, TRAIL-mediated NK cell activation is not limited to its cognate death receptors but also decoy receptor I, adding a new perspective to the perceived regulatory role of decoy receptors in TRAIL-mediated cytotoxicity. Based on these findings, we propose that TRAIL not only contributes to the anti-HIV-1 activity of NK cells but also possesses a multifunctional role beyond receptor-mediated induction of apoptosis, acting as a regulator for the induction of different effector functions.

Keywords death receptor; degranulation; HIV-1; NK cell; TRAIL
Subject Categories Autophagy & Cell Death; Immunology; Microbiology, Virology & Host Pathogen Interaction
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Introduction

Natural killer (NK) cells are innate immune cells which play a pivotal role in antiviral immunity and tumor surveillance (Jost & Altfeld, 2013; Malmberg *et al*, 2017). NK cells exhibit various effector functions, including direct cellular cytotoxicity and production of pro-inflammatory cytokines, to eliminate potential target cells and to shape adaptive immune responses (Vivier *et al*, 2008; Prager & Watzl, 2019). NK cells utilize a large array of germline-encoded surface receptors to interact with their environment and to recognize virus-infected or transformed cells (Biassoni & Malnati, 2018). Multiple factors, including host genetics and differentiation stage, impact the receptor repertoire of NK cells, ultimately leading to a remarkable diversity of NK cells within and across individuals (Horowitz *et al*, 2013; Schwane *et al*, 2020). The integration of activating and inhibitory signaling through these receptors tightly regulates NK cell activity (Long *et al*, 2013). The specific receptor profile of an individual NK cell therefore determines the activation threshold of a given cell and impacts the ability to recognize potential target cells.

Given the importance of NK cells in the early control of viral infections, our group has been investigating the contribution of receptors to an effective antiviral response of NK cells, in particular in HIV-1 infection (Fadda *et al*, 2012; Körner *et al*, 2014, 2017). NK cells contribute to the intrinsic control of HIV-1 infection through NK-cell-mediated immune pressure (Alter *et al*, 2011; Hölzemer *et al*, 2015). Several NK cell receptors have been attributed to promote NK-cell-mediated control of HIV-1 infection and delay progression to AIDS, most prominent receptors that bind to specific HLA class I molecules (Martin *et al*, 2002, 2007). Yet, our understanding about receptor profiles that enable NK cells to effectively recognize HIV-1-infected cells independent of the host's

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HLA class I background is incomplete. We developed an unbiased and comprehensive screening approach that combined a functional readout (degranulation; Alter *et al*, 2004) and the individual assessment of 327 surface antigens on NK cells in co-culture with autologous HIV-1-infected cells. As a result, we identified the Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL) to be associated with increased degranulation of NK cells after exposure to infected cells.

TRAIL is well-known for its role in receptor-mediated cytotoxicity, directly inducing apoptosis on target cells upon receptor engagement (Wiley *et al*, 1995; Pitti *et al*, 1996). TRAIL has multiple soluble and cell surface interaction partners that differ in their subsequent signaling. The death receptors 4 (DR4, TRAIL-R1) and 5 (DR5, TRAIL-R2) contain cytoplasmic death domains that are capable of initiating cell death through the extrinsic apoptotic pathway (Pan *et al*, 1997b; Sheridan *et al*, 1997). In contrast, two so-called decoy receptors, DcR1 and DcR2, lack functional death domains and are therefore not able to trigger the apoptotic cascade (Degli-Esposti *et al*, 1997a; Pan *et al*, 1997a; Sheridan *et al*, 1997). Finally, TRAIL is able to bind osteoprotegerin (OPG), a soluble protein that has been attributed to inhibit osteoclastogenesis and to increase bone density *in vivo* (Emery *et al*, 1998). TRAIL is expressed on a fraction of peripheral blood NK cells and other immune cells and can be induced through various cytokines (Fanger *et al*, 1999; Griffith *et al*, 1999; Kayagaki *et al*, 1999a; Sato *et al*, 2001). TRAIL-induced apoptosis has been implicated to play a role in HIV-1 pathogenesis (Gougeon & Herbeuval, 2012). In this study, we identified TRAIL to be involved in the induction of NK cell degranulation, therefore independent of receptor-mediated cytotoxicity, and further investigated the underlying mechanisms.

Results

NK cells recognizing HIV-1-infected CD4 T cells display elevated TRAIL surface expression

We initially sought to identify NK cell receptors that are involved in the recognition of HIV-1-infected CD4 T cells. For this, we used a large-scale, flow cytometry-based screening approach to simultaneously assess NK cell degranulation [expression of CD107a (Alter *et al*, 2004)] and 327 additional individual surface antigens on primary human NK cells after incubating them with autologous *in vitro* HIV-1-infected CD4 T cells. For subsequent analyses, two subsets were defined, responsive $CD107a^+$ NK cells and non-responsive $CD107a^-$ NK cells (Fig 1A). As shown in Fig 1B, median percentage of $CD107a^+$ NK cells was higher after exposure to HIV-1-infected CD4 T cells (13.8%) than in the presence of mock-infected CD4 T cells (6.9%) or in the absence of target cells (3.4%). Normalization of the individual values to their corresponding mock control showed an HIV-1-specific response for all tested donors ($n = 19$, $P < 0.0001$), ranging from 0.9 percentage points (p.p.) up to 20.7 p.p. (Fig 1C). Analyzing 327 surface antigens, TRAIL was one of 48 molecules that were differentially expressed between the $CD107a^+$ and $CD107a^-$ NK cell subsets (TRAIL: $P < 0.0001$; Fig 1D). The relative frequency of $TRAIL^+$ NK cells was consistently higher in $CD107a^+$ NK cells than in their $CD107a^-$ counterparts, with a median intra-donor difference of 15.1 p.p. ($P = 0.001$, Fig 1E and F). Similar results were observed when median fluorescence intensity (MdFI) was used as an additional metric for TRAIL expression (Fig 1G), with a 2.2-fold higher MdFI in the responsive NK cell subset ($P = 0.001$). These data demonstrated that degranulation of NK cells in response to autologous HIV-1-infected CD4 T cells was associated with increased TRAIL expression. Based on this observation, we further investigated possible underlying causes and postulated the following three hypotheses: (i) TRAIL acts as an activation marker, being upregulated during or after degranulation; (ii) TRAIL is simply co-expressed on NK cell subsets with inherently higher antiviral activity but not involved in the induction of degranulation; and (iii) TRAIL is either directly or indirectly involved in degranulation.

TRAIL is neither an activation marker nor simply co-expressed on antiviral NK cells

In order to investigate whether TRAIL acted as an activation marker or was independently co-expressed in degranulating NK cells, we repeated the previous experimental setup with 13 different healthy donors. In this workflow, we additionally measured TRAIL surface expression on NK cells in response to the presence of mock-infected CD4 T cells or in the absence of target cells. Furthermore, we acquired an increased number of cells for each condition to obtain sufficient cell numbers for high resolution of NK cell subsets. As shown in the left panel of Fig 2A, exposure to HIV-1-infected CD4 T cells yielded in the highest response rate (median 16.4% $CD107a^+$ cells), followed by the condition with mock-infected CD4 T cells (8.1%). In the absence of target cells, only 2.2% of bulk NK cells were $CD107a^+$. Stratification of bulk NK cells into CD56Dim and CD56Bright cells (middle panel) or into un-, KIR-, or NKG2A-educated NK cells (right panel) showed a similar hierarchy of NK cell degranulation for each subset. Comparison of these predefined groups in terms of their antiviral capacity revealed a differential ability to degranulate in response to HIV-1-infected target cells (Fig 2B). CD56Bright NK cells exhibited a higher HIV-1-specific response (15.1 p.p., median) than CD56Dim NK cells (9.4 p.p., $P = 0.002$). Stratification based on education status, showed that HIV-1-specific responses were highest in NKG2A-educated NK cells (14.5 p.p., median). In contrast, KIR-educated NK cells (4.8 p.p.) performed rather poorly compared to NKG2A-educated ($P < 0.001$) or uneducated NK cells (9.7 p.p., $P = 0.005$). Next, we investigated whether TRAIL was upregulated after exposure to target cells (Fig 2C). For all tested subsets, surface density of TRAIL on NK cells exposed to HIV-1-infected CD4 T cells remained unchanged compared to NK cells cultured in the absence of target cells (Bulk: $P = 0.5$; Dim: $P > 0.99$; Bright: $P = 0.1$; Uneducated: $P > 0.99$; KIR-edu.: $P > 0.99$; NKG2A-edu.: $P > 0.99$). Finally, we compared TRAIL surface expression between $CD107a^+$ and $CD107a^-$ NK cells across the previously defined subsets. As shown in Fig 2D, in all the investigated subsets, expression of TRAIL was significantly higher on $CD107a^+$ NK cells (Bulk: $P < 0.001$; Dim: $P < 0.001$; Bright: $P < 0.001$; Uneducated: $P < 0.001$; KIR-edu.: $P < 0.01$; NKG2A-edu.: $P < 0.001$). The fold difference in TRAIL expression between responsive and non-responsive NK cells was not different between

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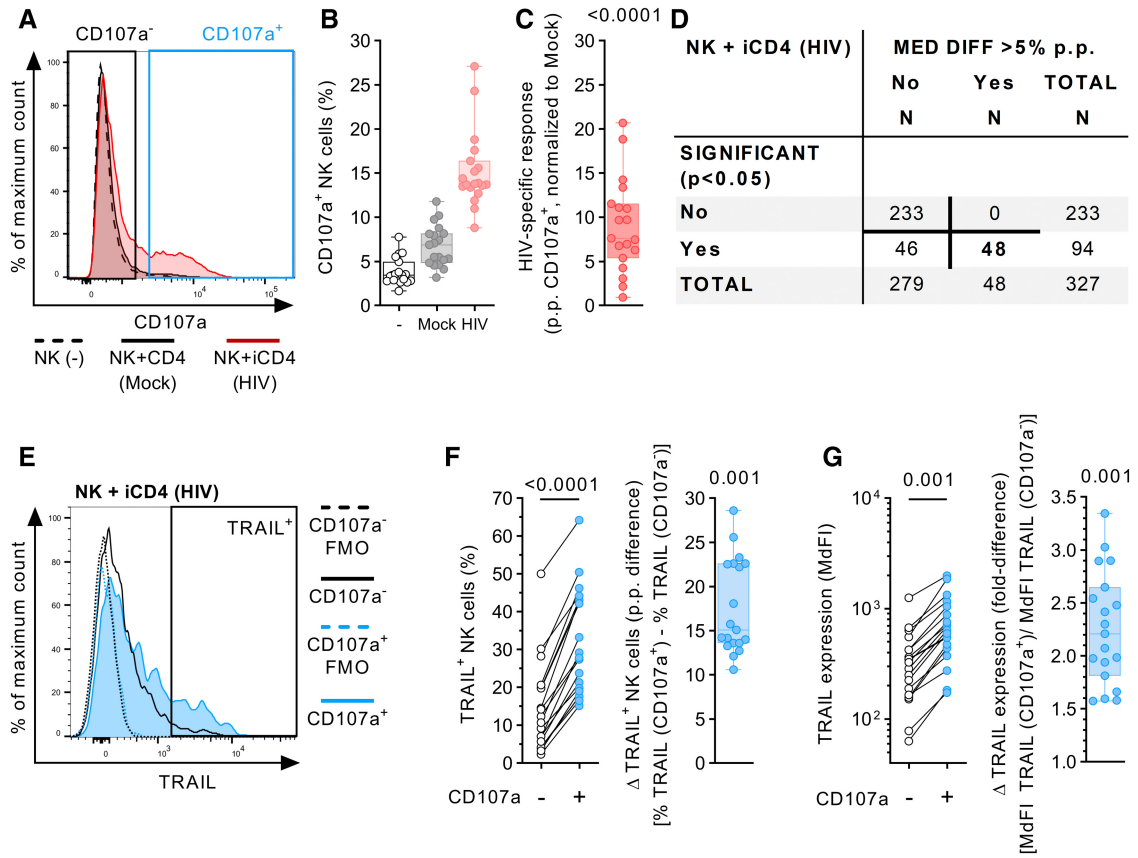

Figure 1. NK cell degranulation is associated with increased TRAIL surface expression after co-incubation with autologous HIV-1-infected CD4 T cells.

Primary human NK cells (n = 19 different donors per condition) were co-incubated with either no target cells (NK(−)), autologous CD4 T cells (Mock), or enriched *in vitro* HIV-1-infected (NL4-3) CD4 T cells (HIV) at an effector:target cell ratio of 1:2. NK cell degranulation was quantified by measuring CD107a surface expression using flow cytometry.

A Representative histograms (overlay) displaying CD107a expression on NK cells after culture with the described culture conditions (NK(−), Mock, HIV). For the HIV-1 co-culture condition, subsequent analyses of antigen expression were conducted by gating non-responsive ($CD107a^-$) and responsive ($CD107a^+$) NK cell subsets.

B Relative frequency of $CD107a^+$ NK cells (y-axis) after culture in the described conditions (x-axis) (n = 19 different donors).

C HIV-specific response of bulk NK cells displayed as percentage points (p.p.) $CD107a^+$ NK cells after normalization to mock CD4 T cells (y-axis) (n = 19 different donors).

D Summary table showing numeric results of the 327 surface antigens analyzed. A total of 48 surface molecules showed statistically significant intra-donor differences > 5 p.p. in expression between $CD107a^+$ and $CD107a^-$ NK cells after exposure to HIV-1-infected CD4 T cells (HIV).

E Representative histograms (overlay) showing TRAIL surface expression as fluorescence intensity on NK cells after co-incubation with HIV-1-infected CD4 T cells. Histograms show TRAIL expression for $CD107a^+$ and $CD107a^-$ NK cell subsets (solid lines) as well as their corresponding FMO controls (dotted lines). Gate defining $TRAIL^+$ cells is indicated as well.

F Left panel: Relative frequency of $TRAIL^+$ cells (y-axis) of $CD107a^+$ and $CD107a^-$ NK cell subsets (x-axis) after co-incubation with HIV-1-infected CD4 T cells. Right panel: Difference in relative frequency of $TRAIL^+$ cells (y-axis) between $CD107a^+$ and $CD107a^-$ cells displayed as p.p. (n = 19 different donors).

G Left panel: TRAIL surface expression (y-axis) of $CD107a^+$ and $CD107a^-$ NK cell subsets (x-axis) after co-incubation with HIV-1-infected CD4 T cells displayed as median fluorescence intensity. Right panel: Differences in TRAIL surface expression (y-axis) between $CD107a^+$ and $CD107a^-$ cells displayed as fold difference (n = 19 different donors).

Data information: Wilcoxon signed-rank test. Adjustment for multiplicity: Benjamini and Hochberg false discovery rate (FDR) (D; F, left panel); Bonferroni (F, right panel; G). Box plots represent the median and 25%/75% percentile. Whiskers indicate the minimum and maximum data points. Lines connecting $CD107a^-$ and $CD107a^+$ cells refer to measured values of the same donor.
Source data are available online for this figure.

CD56Bright (1.46) and CD56Dim NK cells (1.68, P = 0.17; Fig 2E). For the KIR-educated subset, however, median fold difference was significantly lower (1.25) compared to the uneducated (1.49, P = 0.004) or NKG2A-educated subsets (2.05, P = 0.002). Taken together, our data indicated that TRAIL is neither an activation marker in this experimental setting nor simply co-expressed in subsets with inherently higher anti-HIV activity. Instead, in all investigated subsets, NK cell degranulation was associated with increased TRAIL expression, indicating a potential role for the induction of degranulation.

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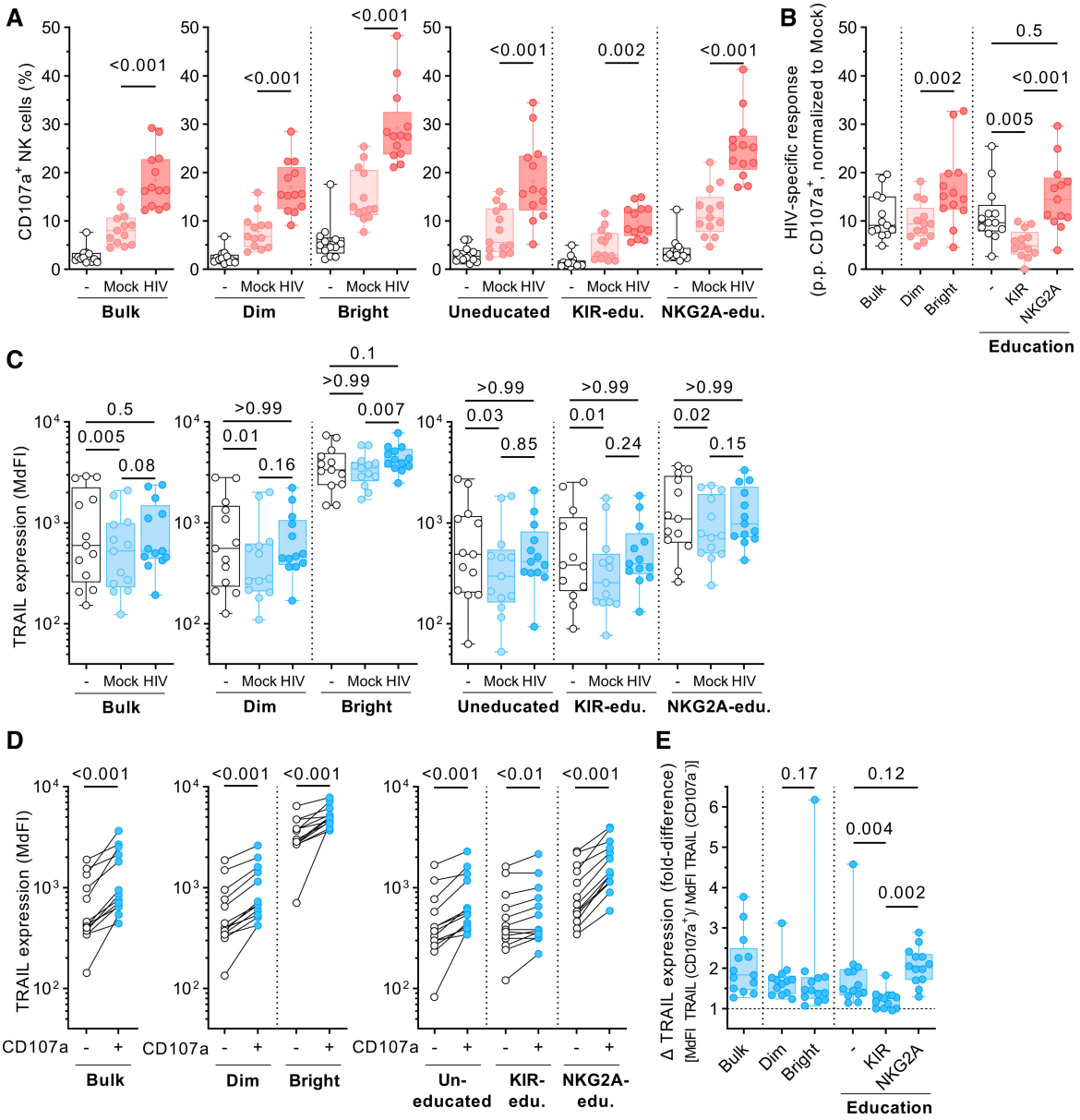

Figure 2. NK cell degranulation is associated with increased TRAIL expression across multiple NK cell subsets.

Comparison of CD107a and TRAIL expression levels between various NK cell subsets after co-incubation with no target cells present (–), autologous CD4 T cells (Mock), or enriched *in vitro* HIV-1-infected (NL4-3) CD4 T cells (HIV) (n = 13 different donors per condition). Predefined NK cell subsets comprise CD56Dim, CD56Bright, and KIR-educated cells, defined as expressing at least one self-inhibitory KIR (2DL1/L2/L3, 3DL1), and negative for NKG2A, NKG2A-educated cells, expressing NKG2A, but lacking self-inhibitory KIR, and uneducated cells, lacking self-inhibitory KIR and NKG2A altogether.

A Relative frequency of $CD107a^+$ cells (y-axis) of predefined NK cell subsets (x-axis). Left panel displays data for bulk NK cells, middle panel for CD56Dim and CD56Bright NK cells, right panel for uneducated, KIR-educated, and NKG2A-educated cells (n = 13 different donors).

B HIV-specific responses of predefined NK cell sub-populations (x-axis) displayed as percentage points (p.p.) $CD107a^+$ NK cells after normalization to mock CD4 T cells (y-axis) (n = 13 different donors).

C TRAIL expression levels (y-axis) of predefined NK cell subsets (x-axis). Left panel displays data for bulk NK cells, middle panel for CD56Dim and CD56Bright NK cells, and right panel for uneducated, KIR-educated, and NKG2A-educated cells. Expression is displayed as median fluorescence intensity (MdFI) (n = 13 different donors).

D Comparison of TRAIL expression levels (y-axis) between $CD107a^-$ and $CD107a^+$ cells of predefined NK cell subsets. Left panel displays data for bulk NK cells, middle panel for CD56Dim and CD56Bright NK cells, right panel for uneducated, KIR-educated, and NKG2A-educated cells. Expression is displayed as MdFI (n = 13 different donors).

E Fold differences in TRAIL surface expression (y-axis) between $CD107a^+$ and $CD107a^-$ cells of predefined NK cell sub-populations (x-axis) (n = 13 different donors).

Data information: Wilcoxon signed-rank test adjusted for multiple comparisons (Bonferroni). Box plots represent the median and 25%/75% percentile. Whiskers indicate the minimum and maximum data points. Lines connecting $CD107a^-$ and $CD107a^+$ cells refer to measured values of the same donor.
Source data are available online for this figure.

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In vitro HIV-1-infected CD4 T cells display increased surface expression of the TRAIL receptors DR4/DR5

Next, we investigated whether TRAIL was directly and/or indirectly involved in the induction of NK cell degranulation. A prerequisite for this hypothesis is the presence of TRAIL receptors on HIV-1-infected target cells that would engage TRAIL on NK cells and subsequently lead to degranulation. Therefore, we assessed the surface expression levels of two interaction partners of TRAIL, the death receptors 4 and 5 (DR4/5) on CD4 T cells. Exemplary histograms in Fig 3A show the combined expression of DR4/5 on mock CD4 T cells or after infection with five different HIV-1 strains (NL4-3, JR-CSF, CH198, CH236, and WITO). Death receptors 4 and 5 are expressed on uninfected CD4 T cells (Mock) as well as on CD4 T cells that are positive for CD4 and negative for the intracellular HIV protein p24 ($CD4^+/p24^-$). DR4/5 expression was increased on HIV-I-infected CD4 T cells ($CD4^-/p24^+$) compared to their $CD4^+/p24^-$ counterparts. As summarized in Fig 3B, exposure to all tested HIV-1 strains resulted in an increase in DR4/5 expression on infected cells, measured as relative fluorescence intensity (RFI).

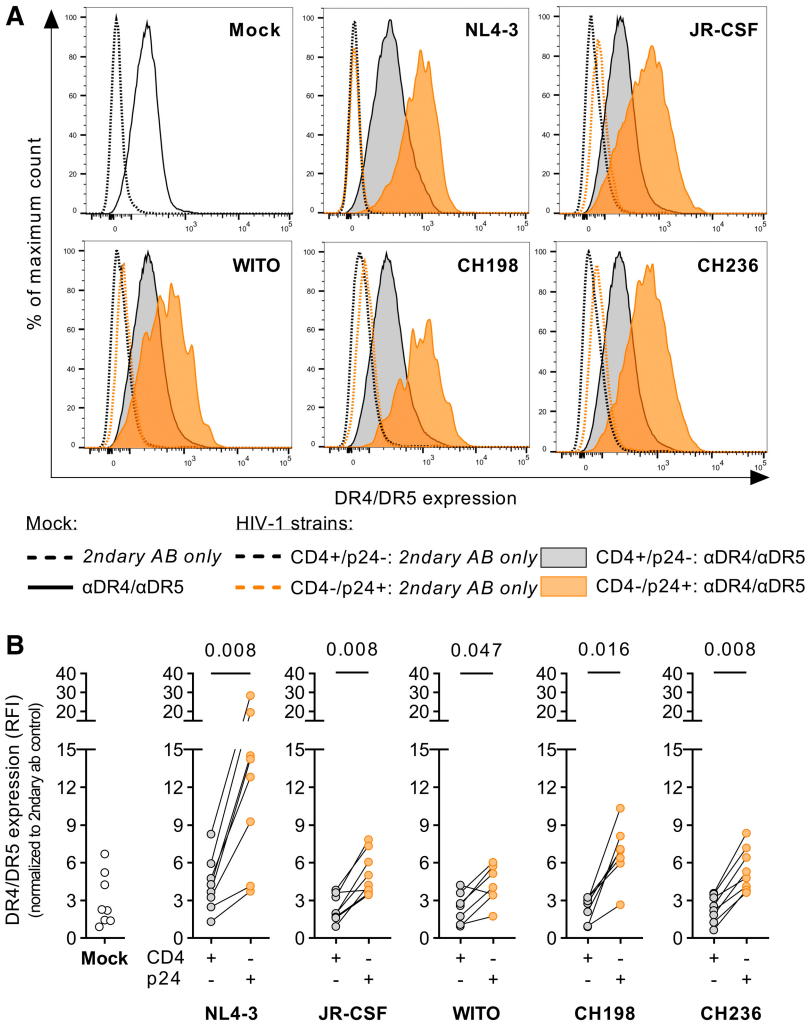

Figure 3. ***In vitro*** **HIV-1-infected CD4 T cells display increased surface expression of the TRAIL receptors DR4/5.**

Primary human CD4 T cells from eight different donors (n = 8) were separately infected with lab-adapted and primary HIV-1 strains. Combined expression of DR4 and DR5 was assessed by flow cytometry 4 days after infection. Uninfected CD4 T cells were determined as CD4-positive and HIV-1 p24-negative, infected cells as CD4-negative and p24-positive.

A Histograms (overlay) of one representative donor displaying combined DR4/5 surface expression on CD4 T cells previously incubated with NL4-3, JR-CSF, WITO, CH198, or CH236. Expression is displayed as fluorescence intensity (x-axis).

B Comparison of the combined surface expression of DR4/5 between mock (white circles), uninfected (grey circles), and infected CD4 T cells (orange circles), from left to right: Mock: n = 8; NL4-3: n = 8; JR-CSF: n = 8; WITO: n = 7; CH198: n = 7; CH236: n = 8 (7–8 different donors per condition). Expression is displayed as relative fluorescence intensity (RFI) after normalization to the respective secondary AB-only control (y-axis).

Data information: Wilcoxon signed-rank test. Values for non-infected and infected cells of the same donor are connected with lines.
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Upregulation was independent of the tropism (X4 vs. R5), subtype (clade B vs. C of group M) or origin (cell culture-adapted vs. primary strains). Of note, uninfected as well as infected cells also showed expression of TRAIL-R3, which is thought to serve as a decoy receptor (DcR1) for TRAIL (Fig EV1). These results showed that interaction partners for TRAIL were present on CD4 T cells and that DR4 and DR5 were even further upregulated on HIV-1-infected target cells.

Antibody-mediated blocking of TRAIL inhibits NK cell degranulation

Then, we investigated whether blocking the interaction between TRAIL and its receptors would affect NK cell degranulation. For this, we co-cultured NK cells with the MHC-class I devoid target cell line 721.221 or autologous HIV-I-infected CD4 T cells, either in the absence or presence of a mouse anti-human TRAIL antibody or the respective antibody isotype. As displayed in Fig 4A, co-culture with 721.221 induced degranulation in up to 41% of NK cells (median, 25.7%). Presence of the isotype antibody did not affect levels of degranulation (26.2%, $P = 0.84$ vs. .221 alone; median inhibition, 1.4%), while pre-incubation with αTRAIL led to a reduced number of CD107a$^+$ NK cells (15.5%, $P = 0.004$ vs. .221 alone) with a median inhibition of 35.4% ($P = 0.002$ vs. isotype, Fig 4B). Similar results, but less pronounced, were observed when we used autologous HIV-1-infected CD4 T cells as target cells (Fig 4C). Here again, presence of the isotype antibody did not affect levels of degranulation ($P > 0.99$ vs. HIV alone; median inhibition 1.7%), while pre-

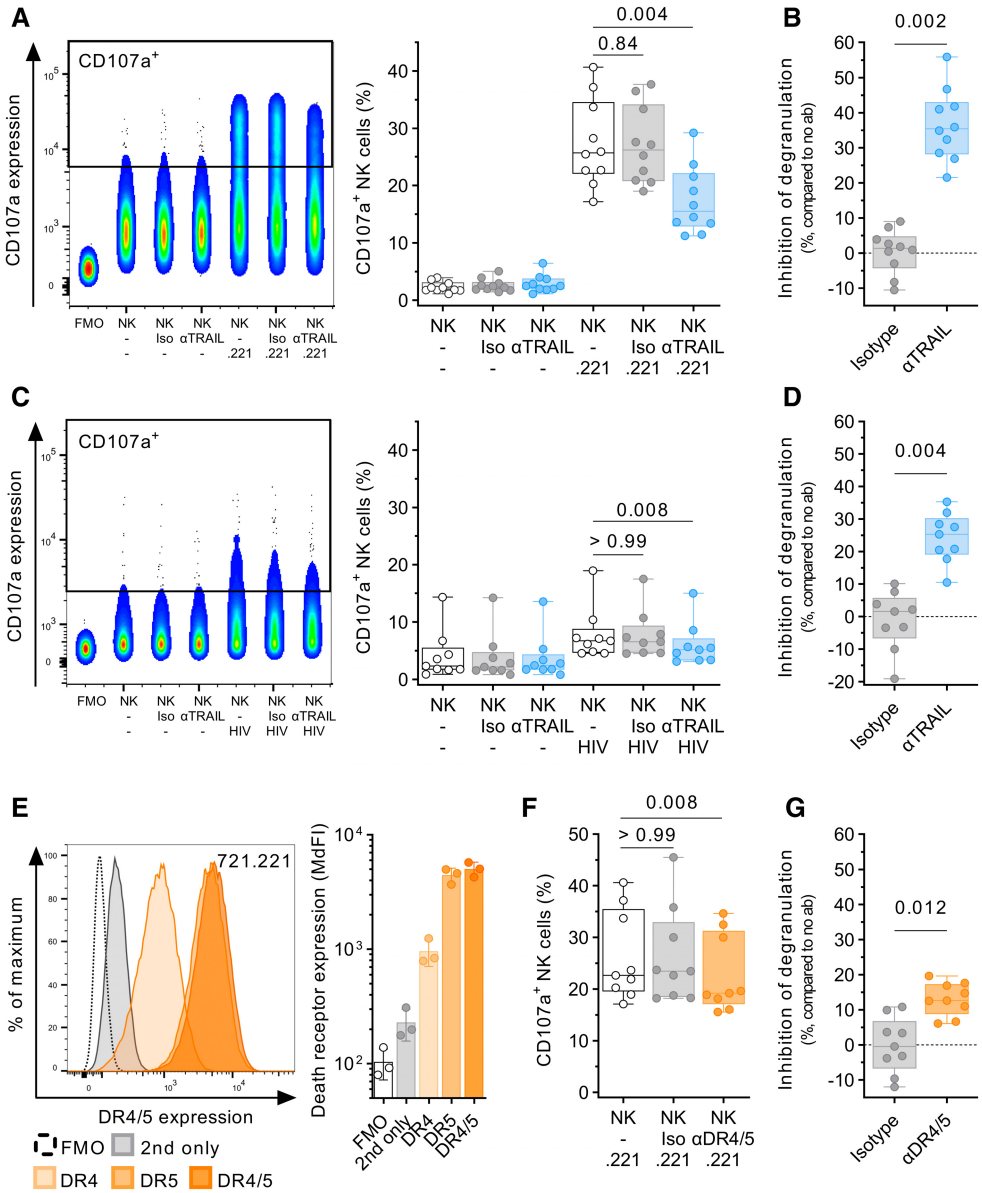

Figure 4.

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Figure 4. Antibody-mediated blocking of TRAIL inhibits NK cell degranulation.

Degranulation of primary human NK cells after co-culture with various target cells in the presence or absence of αTRAIL or αDR4/5.

A Comparison of CD107a expression after co-culture with 721.221 target cells with either 10 μg/ml αTRAIL or isotype control (Iso) using flow cytometry (n = 10 different donors per condition). Each data point represents the mean of two technical replicates. Effector:target ratio was 1:1. Left panel: Concatenated density plot depicting CD107a expression as fluorescence intensity for one representative donor. Right panel: Box plots showing relative frequency of $CD107a^+$ NK cells (y-axis).

B Box plots display inhibition of degranulation (y-axis) after co-culture with 721.221 target cells in presence of either isotype or αTRAIL as relative reduction compared to no antibody (n = 10 different donors per condition).

C Comparison of CD107a expression after co-culture with autologous HIV-1-infected CD4 T cells with either 10 μg/ml αTRAIL or isotype control (Iso) using flow cytometry (n = 9 different donors per condition). Each data point represents the mean of two technical replicates. Left panel: Concatenated density plot depicting CD107a expression as fluorescence intensity for one representative donor. Right panel: Box plots showing relative frequency of $CD107a^+$ NK cells (y-axis).

D Box plots display inhibition of degranulation (y-axis) after co-culture with autologous HIV-1-infected CD4 T cells in the presence of either isotype or αTRAIL as relative reduction compared to no antibody (n = 9 different donors per condition).

E Representative histograms (overlay, left panel) and bar graphs (n = 3 independent experiments, right panel) showing the individual and combined surface expression of DR4 and DR5 on 721.221 cells. Each data point represents the mean of three technical replicates.

F Comparison of CD107a expression after co-culture with 721.221 target cells in the presence of either αDR4/5 (10 μg/ml each) or 20 μg/ml isotype control (Iso) using flow cytometry (n = 9 different donors per condition). Effector:target ratio was 1:1. Box plots showing relative frequency of $CD107a^+$ NK cells (y-axis).

G Box plots display inhibition of degranulation after co-culture with 721.221 target cells in the presence of either isotype or αDR4/5 as relative reduction compared to no antibody (n = 9 different donors per condition).

Data information: Wilcoxon signed-rank test. Adjustment for multiple comparisons was performed using Bonferroni. Box plots represent the median and 25%/75% percentile. Whiskers indicate minimum and maximum data points. Bar graphs represent the mean and the associated whiskers display the SD.
Source data are available online for this figure.

incubation with αTRAIL led to a median inhibition of 25.4% (P = 0.008 vs. HIV alone; P = 0.004 vs. isotype, Fig 4D). Next, we tested whether blocking of the TRAIL receptors DR4/5 on target cells would impact NK cell degranulation. 721.221 cells express both DR4 and DR5 to a measurable degree on the cell surface (Fig 4E). Pre-incubation of 721.221 with DR4/5 antibodies and subsequent co-culture with NK cells led to reduced degranulation in NK cells from all nine tested donors (median, 22.7% vs. 19.2%, P = 0.008 vs. .221 alone; Fig 4F), while pre-incubation with the respective isotype control (median, 23.5%, P > 0.99 vs. .221 alone) had no effect on the frequency of $CD107a^+$ NK cells (median inhibition, −0.4% vs. 12.6%, P = 0.012; Fig 4G). These results showed that abrogation of TRAIL binding to two of its receptors negatively impacted NK cell degranulation, indicating that TRAIL is involved in the degranulation process.

TRAIL engagement directly induces NK cell degranulation

Next, we sought to induce NK cell degranulation through direct engagement of TRAIL. For this, we immobilized (coated) TRAIL antibodies as well as proteins of the different TRAIL receptors on non-tissue culture-treated plates, and then cultured NK cells in the coated wells. Plate-coated αNKG2D and αNKp46 served as positive controls and readily induced degranulation of NK cells in a dose-dependent manner. Similarly, incubation in αTRAIL-coated wells also resulted in a significant increase in $CD107a^+$ NK cells compared to the isotype control (P < 0.001 for all concentrations; Fig 5A). To mimic more physiological conditions, we used proteins of the TRAIL receptors DR4 and DR5 to trigger NK cell degranulation. Human IgG served as positive control through crosslinking of CD16 and strongly induced degranulation (10 μg/ml: median 77.9% $CD107a^+$ cells). Both, DR4 and DR5 likewise induced degranulation of NK cells compared to the uncoated negative control (PBS), although DR5 to a lesser extent (PBS: 1.7%, DR4: 1 μg/ml: 4.2%, 5 μg/ml: 14.3%, 10 μg/ml: 13.9%; DR5: 1 μg/ml: 4.6%, 5 μg/ml: 6.7%, and 10 μg/ml: 5.7%, P = 0.002 for all conditions; Fig 5B). In addition to measuring the percentage of $CD107a^+$ NK cells, we also quantified the release of granzyme B into the supernatant (Fig 5C). NK cell cultures in αTRAIL and αNKp46-coated wells (10 μg/ml) showed increased concentrations of granzyme B in the supernatant

Figure 5. TRAIL engagement directly induces NK cell degranulation.

Degranulation of primary human NK cells after incubation with plate-coated antibodies or whole proteins.

A Comparison of CD107a expression after incubation in either uncoated wells (PBS) or wells coated with αTRAIL, αNKG2D, αNKp46, or isotype using flow cytometry (n = 12 different donors per condition). Left panel: Concatenated density plot depicting CD107a expression as fluorescence intensity (y-axis) for one representative donor and 10 μg/ml antibody concentration. Right panel: Box plots showing relative frequency of $CD107a^+$ NK cells (y-axis) after incubation with plate-coated antibodies of different concentrations (x-axis).

B Comparison of CD107a expression after incubation with plate-coated DR4 protein, DR5 protein, or human IgG using flow cytometry (n = 11 different donors per condition). Left panel: Concatenated density plot depicting CD107a expression as fluorescence intensity (y-axis) for one representative donor and 10 μg/ml protein concentration. Right panel: Box plots showing relative frequency of $CD107a^+$ NK cells (y-axis) after incubation with plate-coated proteins of different concentrations (x-axis).

C Comparison of granzyme B release after incubation with various stimuli (10 μg/ml each). Box plots showing granzyme B concentration in the supernatant as determined by ELISA (left panel: n = 8 different donors per condition, right panel: n = 9 different donors per condition).

D Correlation analysis between relative frequency of $CD107a^+$ NK cells and granzyme B concentration (n = 53, data points obtained from A, B, and C, 11 different donors).

E Comparison of CD107a expression after incubation with plate-coated DcR1 protein, osteoprotegerin (OPG), or human IgG using flow cytometry (n = 9 different donors). Box plots showing relative frequency of $CD107a^+$ NK cells (y-axis) after incubation with plate-coated proteins of different concentrations (x-axis).

Data information: Wilcoxon signed-rank test adjusted for multiple comparisons (Bonferroni). Spearman rank analysis. (A, B, C, E) Each data point represents the mean of two technical replicates. Box plots represent the median and 25%/75% percentile. Whiskers indicate minimum and maximum data points.
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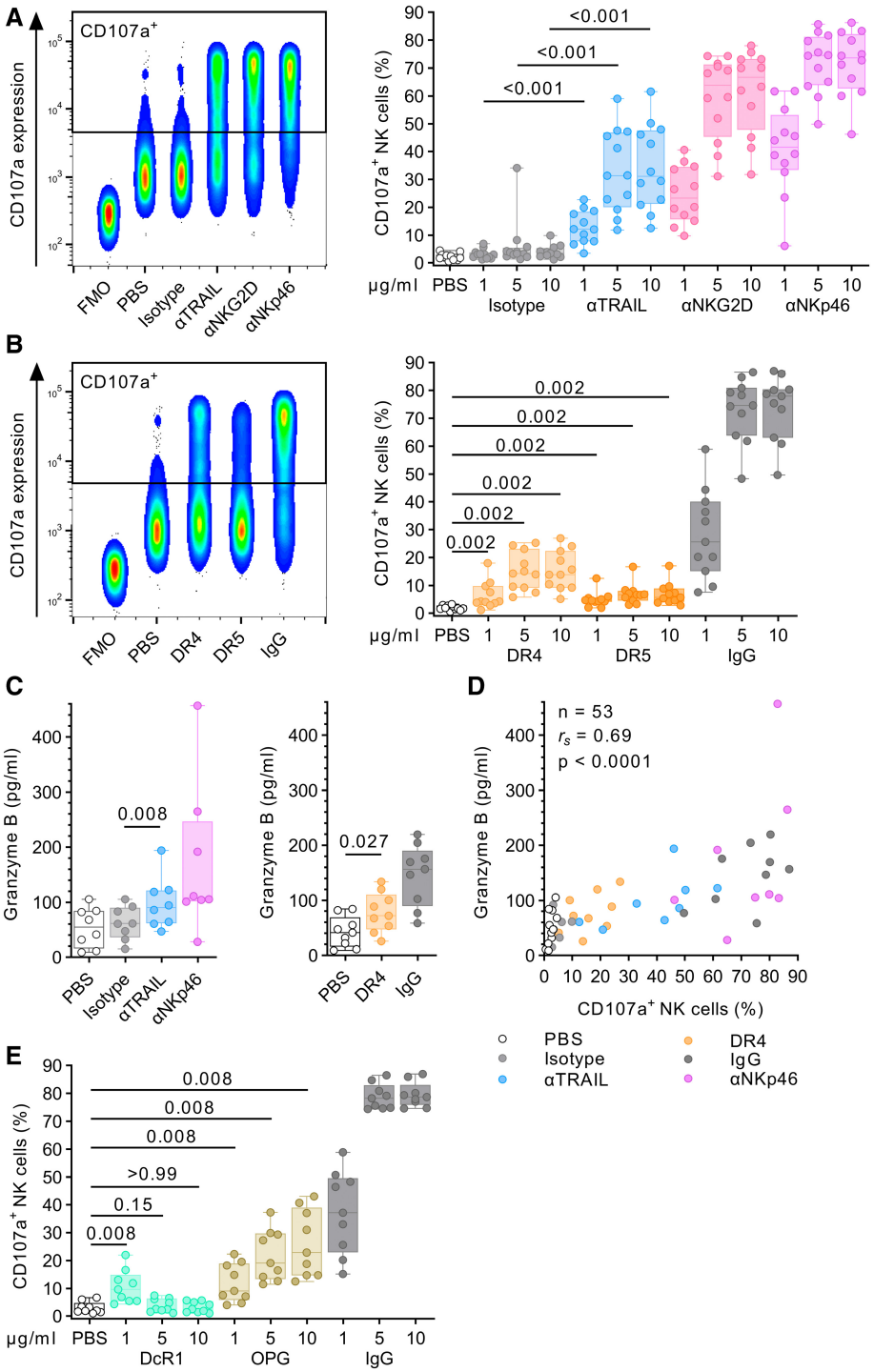

Figure 5.

compared to the controls (αTRAIL vs. Isotype: $P = 0.008$). Increased granzyme B levels were also observed for DR4-coated wells (DR4 vs. PBS: $P = 0.027$). Spearman rank analyses showed a significant correlation between the frequency of CD107a$^+$ NK cells and granzyme B levels in the respective culture supernatants ($r_s = 0.69$, $P < 0.0001$, Fig 5D). Lastly, we were interested whether other known interaction partners of TRAIL are able to trigger NK cell degranulation. NK cells cultured in wells coated with either decoy receptor 1 (DcR1) or OPG induced degranulation in a significant number of NK cells (DcR1 vs. PBS: $P = 0.008$ for 1 µg/ml; OPG vs. PBS: $P = 0.008$ for all tested concentrations; Fig 5E). Of note, DcR1 was the only TRAIL receptor that showed a dose-dependent

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decrease in degranulation, in contrast to OPG, DR4, or DR5. Altogether, our data demonstrated that all tested interaction partners of TRAIL, including decoy receptor I and OPG, triggered degranulation in NK cells; providing further evidence that engagement of TRAIL is able to elicit effector functions in NK cells.

TRAIL engagement induces IFNγ production

Effector functions of NK cells not only comprise granule-mediated cytotoxicity through degranulation but also the production of pro-inflammatory cytokines. Using supernatants collected from plate-coating and TRAIL blocking experiments, we analyzed whether TRAIL interactions may also impact the production of IFNγ. Indeed, NK cells cultured in wells coated with either αTRAIL or DR4 both induced production and release of IFNγ (αTRAIL vs. isotype: $P = 0.008$; DR4 vs. PBS: $P = 0.004$; Fig 6A). In turn, blocking of TRAIL in NK cell: 721.221 co-cultures resulted in reduced concentrations of IFNγ in the supernatant (αTRAIL: $P = 0.016$), while pre-treatment with an isotype antibody had no significant effect on IFNγ production (isotype: $P > 0.99$, median inhibition, −8.2% vs. 32.3%, $P = 0.008$ vs. isotype; Fig 6B). Next, we tested whether the induction of degranulation and IFNγ production was due to an increased ability to adhere to target cells. Quantifying the ability of NK cells to form conjugates with 721.221 cells in the presence of αTRAIL, we did not observe a significant inhibition of conjugate formation (−0.6% vs. 1.9%, $P = 0.148$ vs. Isotype; Fig 6C and D). Finally, we sought to investigate intracellular signaling pathways that may be activated after direct TRAIL engagement and thus may provide evidence for reverse signaling of TRAIL. We examined changes in phosphorylation of the kinases p38 MAPK, Akt, Syk, and the phospholipase PLC-γ2 after direct engagement of TRAIL through immobilized αTRAIL (Fig 6E). All selected molecules are known to be involved in signaling of NKp46, NKG2D, or CD16, which served as positive controls. Phosphorylation occurs rapidly and transiently after receptor engagement with dephosphorylation following shortly thereafter. After an extended incubation time of 30 min, we assessed the changes in phosphorylation of p38 MAPK, Akt, Syk, and PLC-γ2. First, we observed a high baseline phosphorylation of the examined molecules in the majority of NK cells in the unstimulated conditions, PBS (p-Syk: 71.3%, p-p38 MAPK: 71.7%, p-Akt: 69.2%, p-PLC-γ2: 71.1%), and isotype control (p-Syk: 72.6%, p-p38 MAPK: 77.8%, p-Akt: 69.2%, p-PLC-γ2: 65.7%). Second, either engagement of the activating receptors NKp46, NKG2D, or CD16 by immobilized antibodies led to a marked reduction in NK cells expressing phosphorylated signaling proteins after the prolonged incubation time indicating the return of the signaling molecules into their dephosphorylated state (αNKp46: p-Syk: 34.8%, p-p38 MAPK: 56.1%, p-Akt: 33.0%, p-PLC-γ2: 26.6%; αNKG2D: p-Syk: 25.5%, p-p38 MAPK: 31.3%, p-Akt: 21.5%, p-PLC-γ2: 18.3%; αCD16: p-Syk: 28.5%, p-p38 MAPK: 36.8%, p-Akt: 23.1%, and p-PLC-γ2: 22.8%). Observed effects of dephosphorylation after the engagement of TRAIL through immobilized αTRAIL were less pronounced. NK cells co-cultured with αTRAIL did exhibit a noticeable but smaller reduction in phosphorylated Syk^+, Akt^+, and PLC-$y2^+$ but not p38 $MAPK^+$ NK cells (p-Syk: 60.5%, p-p38 MAPK: 75.4%, p-Akt: 62.0%, and p-PLC-γ2: 56.3%). These results seem to reflect the hierarchy of NK degranulation levels we observed after NK cell receptor cross-linking in Fig 5.

Altogether, our results demonstrated that the direct engagement of TRAIL elicits multiple effector functions in NK cells, including IFNγ production. Investigation of the underlying mechanisms was less conclusive but did not rule out intracellular signaling at least for some of the signaling molecules we analyzed. In addition, in our experimental setup, TRAIL engagement had limited effects on cell adhesion.

TRAIL contributes to NK-cell-mediated killing of target cells

Lastly, we investigated the contribution of TRAIL to NK cell cytotoxicity. For this, we conducted multiple killing assays with various target cells (Fig 7). First, we co-cultured 722.221 target cells with NK cells in the presence of αTRAIL or an isotype control and

Figure 6. TRAIL engagement induces Interferon γ production but does not promote cell adhesion.

A Comparison of IFNγ production (*y*-axis) after incubation with plate-coated antibodies or proteins (10 μg/ml each) (*x*-axis). Box plots showing IFNγ concentration in the supernatant as determined by ELISA (left panel: $n = 8$ different donors per condition, right panel: $n = 9$ different donors per condition).

B Comparison of IFNγ levels in the supernatant after co-culture of NK cells with 721.221 target cells in the presence or absence of either 10 μg/ml αTRAIL or isotype control (Iso) using ELISA ($n = 8$ different donors per condition). Effector:target ratio was 1:1. Left panel: Box plots showing IFNγ levels (*y*-axis) for the described culture conditions (*x*-axis). Right panel: Box plots display inhibition of IFNγ production by NK cells (*y*-axis) in presence of either isotype or αTRAIL as relative reduction compared to no antibody.

C Representative contour plots showing conjugate formation between NK cells and 721.221 target cells as determined by flow cytometry. Plots showing three distinct populations at the beginning of the co-culture (0 min) and after 50 min: single 721.221 cells (CT Orange positive), single NK cells (CT Violet positive), and conjugates (double positive). Relative frequency of NK cells in conjugates is calculated based on the total number of gated NK cells.

D Comparison of conjugate formation after co-culture between NK cells and 722.221 cells after 0 min and after 50 min in the presence or absence of 10 μg/ml αTRAIL or isotype control ($n = 8$ different donors). Left panel: Box plots showing relative frequency of NK cells in conjugates (*y*-axis) for different time points and conditions (*x*-axis). Right panel: Box plots display inhibition of conjugate formation (*y*-axis) in the presence of either isotype or αTRAIL as relative reduction compared to no antibody.

E Expression levels of phosphorylated signaling proteins. Upper panel: Concatenated contour plots of one donor depicting the expression of phosphorylated signaling proteins Syk, p38 MAPK, Akt, and PLC-γ2 for the following culture conditions and controls (*x*-axis: left to right): FMO, PBS, isotype, αTRAIL, αNKp46, αNKG2D, and αCD16. Lower panel: Bar graphs showing the percentage of p-Syk^+, p-p38 $MAPK^+$, p-Akt^+, and p-PLC-$\gamma2^+$ NK cells after 30 min of stimulation ($n = 3$ different donors). Bar graphs represent the median, whiskers display minimum and maximum data points.

Data information: Wilcoxon signed-rank test. (A, B, D) Samples were acquired in duplicate and the mean was calculated for each donor and condition. Box plots represent the median and 25%/75% percentile. Whiskers indicate minimum and maximum data points.
Source data are available online for this figure.

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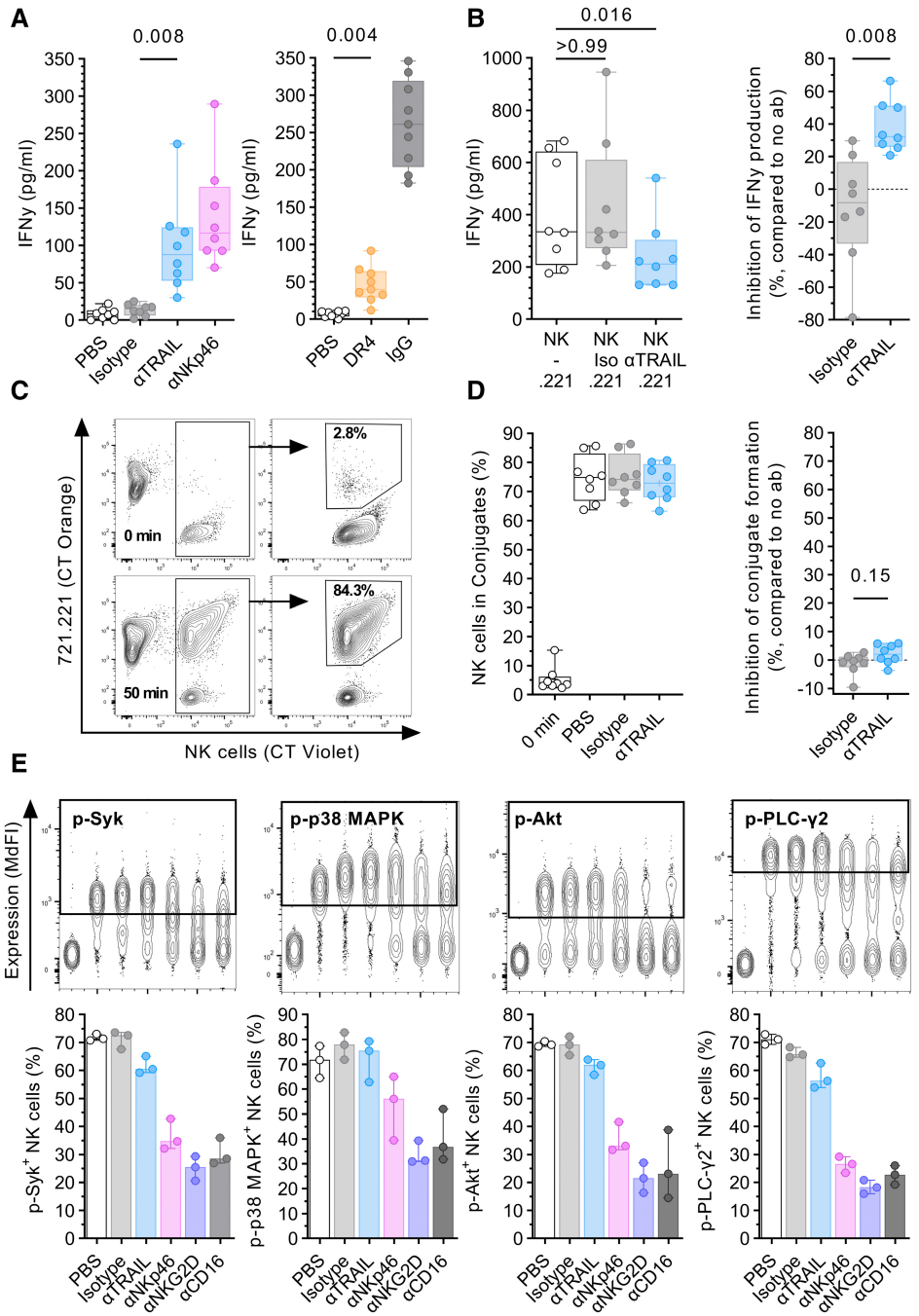

Figure 6.

quantified the remaining cells relative to the number of target cells alone (Fig 7A). NK cells pre-treated with soluble αTRAIL showed a significant reduction in their ability to lyse .221 cells compared to isotype-treated NK cells (median, 18.3% vs. 24.6% remaining cells, $P = 0.012$ vs. isotype). Next, we quantified the preferential killing of target cells by NK cells compared to control cells in competitive killing assays. .221-Cas9 (DR4/5 positive) and Raji cells overexpressing DR5 (Raji-DR5^{++}) served as target cells. The respective control cells were transduced .221 with a DR4 and DR5 gene knockout (.221-DR4/5KO) or Raji-pSIP expressing a lower amount of DR5. Calculation of target:control ratios and the specific lysis of target cells showed that .221-Cas9 cells were preferentially depleted by NK cells (Fig 7B, $P = 0.002$). A similar outcome was observed for Raji cells with elevated DR5 expression (Fig 7C $P = 0.0005$). The results of these experiments showed that the target cells expressing death receptors or higher amounts of death receptors are increasingly sensitive to NK-cell-mediated cytotoxicity.

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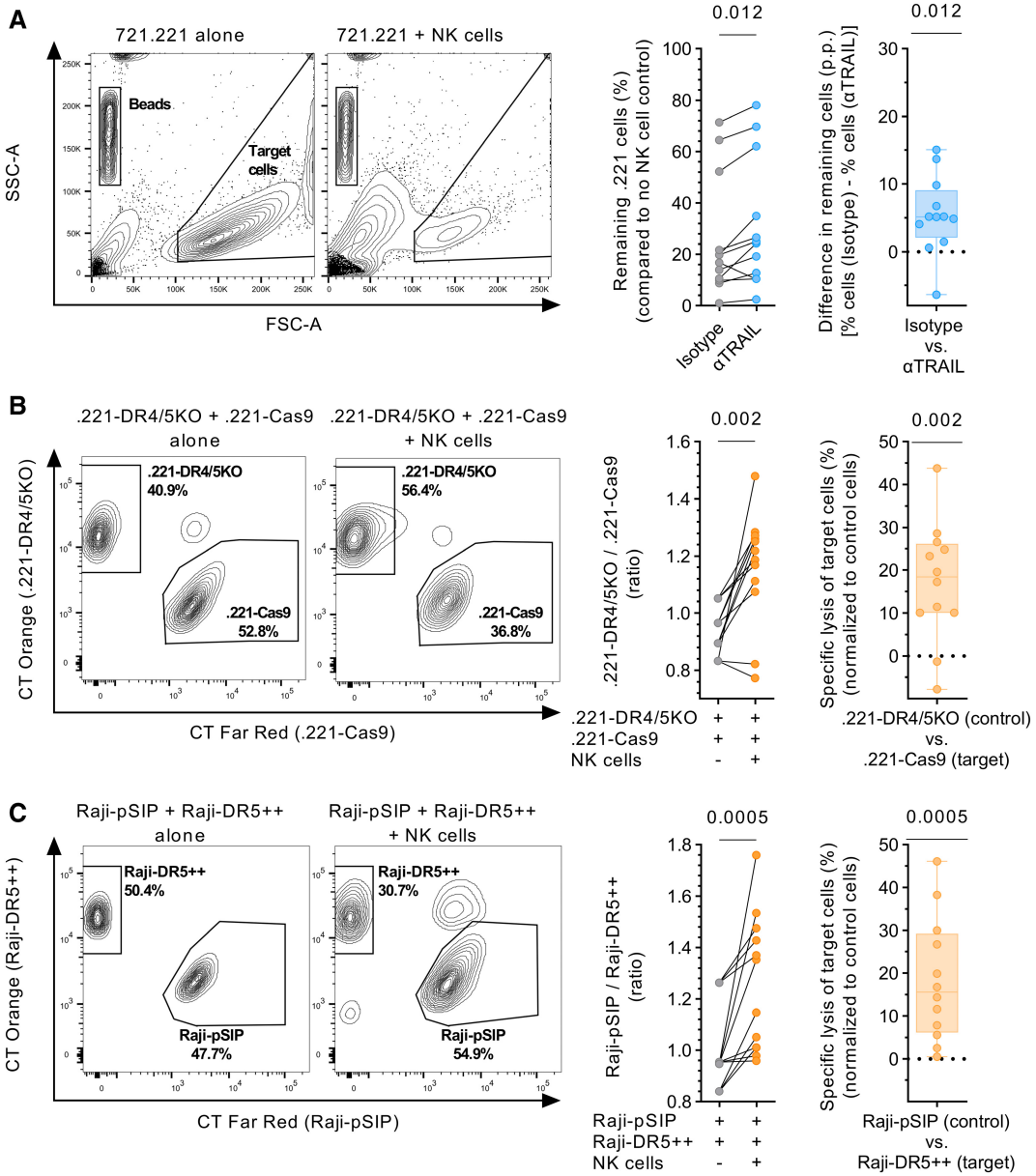

Figure 7. TRAIL contributes to NK-cell-mediated killing of target cells.

Lysis of different target cells in co-culture with NK cells was quantified in various cytotoxicity assays.

A Left panel: Representative contour plots showing depletion of 721.221 target cells in the presence of NK cells. Middle panel: Percentage of target cells remaining (*y*-axis) after co-culture with NK cells in the presence of either αTRAIL or isotype control, in reference to target cells kept alone. Right panel: Box plots displaying difference in target cells remaining (*y*-axis) between αTRAIL and isotype conditions displayed as p.p. (n = 12 different donors). Each data point represents the mean of at least two technical replicates.

B Left panel: Representative contour plots showing the percentage of .221-DR4/5KO (control) and .221-Cas9 cells (target) in the presence or absence of NK cells. Middle panel: Ratio between .221-DR4/5KO and .221-Cas9 cells (*y*-axis) in the presence or absence of NK cells. Right panel: Specific lysis of .221-Cas9 cells displayed as percent (n = 12 different donors). Each data point represents the mean of at least three technical replicates.

C Left panel: Representative contour plots showing the percentage of Raji-pSIP (control) and Raji-DR5^{++} (target) in the presence or absence of NK cells. Middle panel: Ratio between Raji-pSIP and Raji-DR5^{++} (*y*-axis) in the presence or absence of NK cells. Right panel: Specific lysis of Raji-DR5^{++} cells displayed as % (n = 12 different donors). Each data point represents the mean of at least three technical replicates.

Data information: Wilcoxon signed-rank test. Experiments were performed in four batches with three different donors each. "No NK" control samples served as a reference for all donors in each batch. Lines connect each data value of the NK cell condition with their designated "No NK" control. Box plots represent the median and 25%/75% percentile. Whiskers indicate minimum and maximum data points.
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Discussion

In this study, we provide evidence that TRAIL contributes to the anti-HIV-1 activity of NK cells beyond receptor-mediated cytotoxicity through induction of degranulation. Direct engagement of TRAIL elicited multiple effector functions in human NK cells. Both degranulation and IFNγ production in NK cells were triggered by cross-linking TRAIL with either its cognate receptors or αTRAIL, as well as inhibited through antibody-mediated blockade of TRAIL interactions.

The ability of TRAIL to transduce signals after receptor engagement has been debated for a long time with scattered reports on that matter in different species and cell types. In 2001, Chou and colleagues described enhanced proliferation and increased IFNγ production in murine T cells after cross-linking TRAIL through DR4-Fc fusion proteins (Chou *et al*, 2001). However, TRAIL engagement alone was not sufficient to induce proliferation or cytokine production, rather acting as a co-stimulatory molecule in combination with T cell receptor engagement. In a subsequent study, the group conveyed similar findings in human CD4 T cells but not in cytotoxic CD8 T cells (Tsai *et al*, 2004). In TRAIL-deficient mice, murine NK cells showed reduced cytotoxicity but unchanged expression of CD107a after exposure to YAC-1 target cells (Cardoso Alves *et al*, 2020). The same study also reported increased numbers and relative frequencies of IFNγ$^+$ NK cells upon LCMV infection. This and observations by Diehl *et al*. indicated that TRAIL may have an inhibitory role in murine viral infections (Diehl *et al*, 2004). However, these effects may not be linked to reverse signaling but rather caused by other indirect mechanisms during the antiviral immune response, such as altered NK cell–DC cross-talk (Iyori *et al*, 2011). These studies hinted at the ability of TRAIL to induce additional effector functions on the expressing immune cells but remain altogether puzzling; TRAIL only acted as a co-stimulatory molecule in CD4 T cells and not in cytotoxic T cells which are more closely related to NK cells in their role as cytotoxic effectors. Unaltered levels of degranulation in NK cells from TRAIL-deficient mice may indicate differences in TRAIL signaling between mice and humans.

In this study, we showed that blockade of TRAIL interactions resulted in significantly reduced levels of degranulation after exposure to the MHC class I devoid cell line 721.221 and autologous HIV-I-infected CD4 T cells. Similar results were recently described for IL-18/poly I:C-treated NK cells after co-culture with the human hepatic stellate cell (HSC) line LX2 and primary HSCs (Li *et al*, 2019). The authors observed that blockade of TRAIL inhibited the interaction between NK cells and LX2 cells. Our results, however, did not show a significant contribution of TRAIL in conjugate formation between NK cells and 721.221 cells. Nevertheless, our data also demonstrated that blockade of either side of the interaction between TRAIL and its receptors impacts NK cell degranulation. These observations do not rule out the contribution of TRAIL in target cell adhesion and in the formation of an immunological synapse but indicate that its specific contribution may be target cell specific. The presence of TRAIL receptors on the surface of the respective target cell and their ratio to the expression of ligands for other NK cell receptors may be a determining factor.

Cross-linking of TRAIL with proteins of the death receptors 4 and 5 induced degranulation and IFNγ production in NK cells. Beyond its potential role in facilitating target cell adhesion, these observations indicate that TRAIL elicits effector functions in NK cells. Interestingly, this was also the case with other interaction partners of TRAIL, the decoy receptor 1 (DcR1), and osteoprotegerin (OPG). DcR1 (TRAIL-R3) does not contain a death domain and is thus not able to induce apoptosis (Degli-Esposti *et al*, 1997b). Hence, DcRI as well as the related DcR2 (TRAIL-R4) have been attributed to regulate TRAIL sensitivity by sequestering TRAIL in lipid rafts or forming heteromeric complexes with DR5, thus inhibiting caspase-8 activation (Clancy *et al*, 2005; Mérino *et al*, 2006). OPG has been shown to inhibit TRAIL-mediated apoptosis (Emery *et al*, 1998) and has been subsequently implicated to play a role in various malignancies (Zauli *et al*, 2009). In light of our findings, OPG may serve as a double-edged sword, shielding tumor cells from TRAIL-induced apoptosis on the one hand (Holen *et al*, 2002, 2005; Shipman & Croucher, 2003; De Toni *et al*, 2008) and stimulating TRAIL-mediated anti-tumor activity of NK cells (Berg *et al*, 2009) by triggering degranulation on the other. Overall, our results may add a new perspective on the perceived roles of DcR1 and OPG in the context of NK-cell-mediated tumor surveillance.

Although the accumulating evidence of our and previous studies indicates bidirectional signaling of TRAIL, little is known about the underlying signaling pathways. TRAIL is a member of the TNFα superfamily and reverse signaling has been described for some other members (Sun & Fink, 2007; Lee *et al*, 2019). However, TRAIL contains neither an immunoreceptor tyrosine-based activation motif (ITAM) in the short cytoplasmic tail or a positively charged amino acid (lysine or arginine) in the transmembrane domain that could confer association with adapter molecules such as DNAX activation protein 12 (DAP12) or CD3ζ (Humphrey *et al*, 2005; Lanier, 2009). Therefore, the potential signaling pathway may differ from activating receptors like NKG2D, NKp46, or certain KIRs (Campbell *et al*, 1998; Meza Guzman *et al*, 2020). Syk, Akt, and PLC-γ2 play important roles in the signaling pathways of activating NK cell receptors (Spaggiari *et al*, 2001; Jiang *et al*, 2002; Sutherland *et al*, 2002; Upshaw *et al*, 2005). Our assessment of the phosphorylation of these signaling molecules indicated an increased baseline phosphorylation likely associated with prolonged IL-2 and IL-15 treatment, similar to observations in murine NK cells (Luu *et al*, 2021). Further investigations into the participation of these signaling molecules in TRAIL signaling did not yield conclusive results in our experimental setup, which aimed to measure the dephosphorylation after an elapsed signaling cascade. Since Syk and PLC-γ2 enable the activation of signaling pathways leading to the activation of the MAP kinases ERK and JNK (Jiang *et al*, 2002; Chen *et al*, 2007), TRAIL may also utilize one of these MAPKs for signal transduction. In an earlier study, activation of p38 MAPK after TRAIL-induced co-stimulation of CD4 T cells was reported (Tsai *et al*, 2004). Subsequent investigations in CD4 T cells observed phosphorylation of the upstream TCR-proximal tyrosine kinases, Lck, and ZAP70 and activation of the NF-kB pathway after TRAIL co-stimulation (Huang *et al*, 2011). ZAP70 binds to phosphorylated ITAMs in the cytoplasmic domain of activating adapter proteins (Vivier *et al*, 1993; Ting *et al*, 1995; Lanier *et al*, 1998; Augugliaro *et al*, 2003), therefore TRAIL may utilize ITAM-mediated signaling in an indirect manner. Co-localization of TRAIL in nanoscale clusters with other immune receptors and DAP12 is therefore one possibility to hijack adapter molecules for downstream signaling (Oszmiana *et al*, 2016). In this context, it has been shown that CD59 acts as a co-receptor for the activating receptor NKp46 through physical association, promoting

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signaling and subsequent cytotoxicity, despite lacking a signaling domain on its own (Marcenaro *et al*, 2003). So far, the exact mechanism of how TRAIL engagement induces effector functions eventually remains obscure and will require more focused investigations.

We initially observed increased TRAIL expression in NK cells degranulating against HIV-infected cells indicating the involvement of TRAIL in the anti-HIV-1 activity of NK cells. Multiple studies have previously investigated the role of TRAIL and its receptors in immune control and pathogenesis of HIV-1, reviewed in Gougeon and Herbeuval (2012). TRAIL was upregulated in multiple types of immune cells, in HIV-1 infection *in vivo*, *in vitro,* and in animal models (Herbeuval *et al*, 2005a, 2005c, 2009; Hardy *et al*, 2007; Stary *et al*, 2009; Cheng *et al*, 2020). Serum levels of soluble TRAIL were elevated in HIV-1$^+$ individuals and correlated with viral load (Herbeuval *et al*, 2005a). In turn, increased expression of TRAIL receptors on CD4 T cells was observed in HIV-1$^+$ donors (Herbeuval *et al*, 2005b, 2009). In our *in vitro* infection models, we demonstrated that upregulation of DR4/5 was independent of the tested HIV-1 strains. This indicates that upregulation of death receptors is a cellular response of the infected cell which HIV-1 may not be able to prevent through immune evasion mechanisms. Therefore, DR4/5 on infected cells may serve as target molecules for immunotherapeutic HIV-1 cure approaches (Deeks, 2012), enabling elimination of infected cells through TRAIL$^+$ effector cells after reversal of HIV-1 latency. Nevertheless, little is known about TRAIL and NK-cell-mediated induction of apoptosis in HIV-1 infection. One study showed that IL-15-stimulated NK cells from HIV-1-infected donors displayed improved killing of K562 target cells in a TRAIL-dependent manner (Lum *et al*, 2004). In addition, in other viral infections, such as HCV infection, *in vitro* studies indicated that TRAIL$^+$ NK cells may play a crucial part in viral immunity by eliminating virus-replicating cells by TRAIL-mediated cytotoxicity (Stegmann *et al*, 2010). Enhanced TRAIL-mediated cytotoxic activity by NK cells against murine cells infected with EMCV was observed as well (Sato *et al*, 2001). Our data confirmed that TRAIL contributes to NK-cell-mediated cytotoxicity against target cells expressing death receptors recognized by TRAIL, but did not allow the distinction between granule- or death receptor-mediated target cell lysis. Additional experimental models are therefore needed to determine the overall contribution of TRAIL-induced degranulation to NK cell cytotoxicity and in comparison to TRAIL-induced apoptosis via death receptors.

Altogether, our study indicates that TRAIL is involved in NK-cell-mediated anti-HIV-1 activity by directly triggering degranulation. These observations now raise additional questions related to the underlying mechanisms, including the signaling pathway that links initial TRAIL engagement to downstream effector functions. Based on our findings we propose a multifunctional role for TRAIL beyond receptor-mediated cytotoxicity, acting as a regulator for the induction of different effector functions in human NK cells. Hence, TRAIL may remain a target of interest for NK-cell-based immunotherapeutic approaches in cancer or antiviral therapies that would harness the ability of TRAIL to induce NK-cell-mediated target cell killing through multiple avenues.

Materials and methods

Reagents and Tools table

Reagent/Resource	Reference or Source	Identifier or Catalog Number
Experimental Models		
Citrate-treated peripheral blood (*H. sapiens*)	Institute of Transfusion Medicine, University Medical Center Hamburg-Eppendorf, Hamburg, Germany	N/A
EDTA-treated peripheral blood (*H. sapiens*)	Hamburger Gesundkohorte University Medical Center Hamburg-Eppendorf, Hamburg, Germany	N/A
HEK293T/17 (*H. sapiens*)	ATCC	Cat#CRL-11268; RRID:CVCL_1926
LCL 721.221 (*H. sapiens*)	ATCC	Cat#CRL-1855; RRID:CVCL_6263
.221-Cas9	Generated in this study	N/A
.221-DR4/5KO	Generated in this study	N/A
Raji (*H. sapiens*)	Ragon Institute of MGH, MIT and Harvard, Cambridge, MA, USA	RRID:CVCL_0511
Raji-pSIP	Generated in this study	N/A
Raji-DR5^{++}	Generated in this study	N/A
pNL4-3	NIH HIV Reagent Program	Cat#ARP-114
pYK-JRCSF	NIH HIV Reagent Program	Cat#ARP-2708
HIV-1 WITO	Mary Carrington (National Cancer Institute); Ochsenbauer *et al*, 2012	N/A
HIV-1 CH198	Beatrice Hahn (University of Pennsylvania); Parrish *et al*, 2013	N/A
HIV-1 CH236	Beatrice Hahn (University of Pennsylvania); Fenton-May *et al*, 2013	N/A

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Reagents and Tools table (continued)

Reagent/Resource	Reference or Source	Identifier or Catalog Number
Recombinant DNA		
pLVX-SIP	Ragon Institute of MGH, MIT and Harvard, Cambridge, MA, USA; Dr. Thomas Pertel	N/A
pCMV-VSV-G	Addgene	Cat#8454; RRID:Addgene_8454
psPAX2	NIH HIV Reagent Program	Cat#ARP-11348; RRID:Addgene_12260
plentiCas9-Blast	Addgene	Cat#52962; RRID:Addgene_52962
plentiGuide-Puro encoding gRNA sequences	GenScript	Cat#SC1678
Antibodies		
Mouse α-human CD3 PerCP-Cy5.5 (clone UCHT1)	BioLegend	Cat#300430; RRID:AB_893299
Mouse α-human CD3 PE/Dazzle594 (clone UCHT1)	BioLegend	Cat#300450; RRID:AB_2563618
Mouse α-human CD3 BV421 (clone UCHT1)	BioLegend	Cat#300434; RRID:AB_10962690
Mouse α-human CD3 Pacific blue (clone UCHT1)	BD Biosciences	Cat#558117; RRID:AB_397038
Mouse α-human CD4 APC (clone RPA-T4)	BD Biosciences	Cat#555349, RRID:AB_398593
Mouse α-human CD4 APC (clone OKT4)	BioLegend	Cat#317416; RRID:AB_571945
Mouse α-human CD4 BV711 (clone RPA-T4)	BioLegend	Cat#300558, RRID:AB_2564393
Mouse α-human CD14 PerCP-Cy5.5 (clone HCD14)	BioLegend	Cat#325622; RRID:AB_893250
Mouse α-human CD16 BV785 (clone 3G8)	BioLegend	Cat#302046; RRID:AB_2563803
Mouse α-human CD16 (clone 3G8)	BioLegend	Cat#302014; RRID:AB_314214
Mouse α-human CD19 PerCP-Cy5.5 (clone HIB19)	BioLegend	Cat#302230; RRID:AB_2073119
Mouse α-human CD45 AF700 (clone 2D1)	BioLegend	Cat#368514; RRID:AB_2566374
Mouse α-human CD45 BV605 (clone 2D1)	BioLegend	Cat#368524; RRID:AB_2715826
Mouse α-human CD45 PerCP-Cy5.5 (clone 2D1)	BioLegend	Cat#368503; RRID:AB_2566351
Mouse α-human CD56 BUV395 (clone NCAM16.2)	BD Biosciences	Cat#563554; RRID:AB_2687886
Mouse α-human CD57 PE/Dazzle594 (clone HNK-1)	BioLegend	Cat#359620; RRID:AB_2564063
Mouse α-human CD107a BV510 (clone H4A3)	BioLegend	Cat#328632; RRID:AB_2562648
Mouse α-human KIR2DL1/S5 FITC (clone 143211)	R&D Systems	Cat#FAB1844F-100; RRID:AB_2130402
Mouse α-human KIR2DL2/L3/S2 BV711 (clone DX27)	BD Biosciences	Cat#745442; RRID:AB_2742987
Human α-human NKG2C PE (clone REA205)	Miltenyi Biotec	Cat#130-103-635; RRID:AB_2655394
Mouse α-human KIR3DL1 BV421 (clone DX9)	BioLegend	Cat#312714; RRID:AB_2561652
Mouse α-human NKG2A PE-Cy7 (clone Z199)	Beckman Coulter	Cat#B10246; RRID:AB_2687887
Mouse α-human CD253 (TRAIL) APC (clone RIK-2.1)	Miltenyi Biotec	Cat#130-097-314; RRID:AB_2656681
Mouse α-human CD253 (TRAIL) (clone RIK-2)	BioLegend	Cat#308214; RRID:AB_2814155
Mouse α-human TRAIL-R1 (DR4) (clone HS101)	AdipoGen	Cat#AG-20B-0022PF; RRID:AB_2490215
Mouse α-human CD261 (DR4) Biotin (clone DJR1)	Miltenyi Biotec	Cat#130-109-084; RRID:AB_2656741
Mouse α-human TRAIL-R2 (DR5) (clone HS201)	AdipoGen	Cat#AG-20B-0023; RRID:AB_2490218
Mouse α-human TRAIL-R2 (DR5) (clone HS201)	AdipoGen	Cat#AG-20B-0023PF; RRID:AB_2490221
Mouse α-human CD262 (DR5) Biotin (clone DJR2-4)	Miltenyi Biotec	Cat#130-097-303; RRID:AB_2656745
α-human TRAIL-R3 (DcR1, CD263) (polyclonal Goat IgG)	R&D Systems	Cat#AF630; RRID:AB_355488
Mouse α-human CD314 (NKG2D) (clone 1D11)	BioLegend	Cat#320814; RRID:AB_2561488

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Reagents and Tools table (continued)

Reagent/Resource	Reference or Source	Identifier or Catalog Number
Mouse α-human CD314 (NKG2D) PE (clone 1D11)	BioLegend	Cat#320806; RRID:AB_492960
Mouse α-human CD335 (NKp46) (clone 9E2)	Miltenyi Biotec	Cat#130-094-271; RRID:AB_10828456
Mouse α-human CD335 (NKp46) FITC (clone 9E2)	BioLegend	Cat#331922; RRID:AB_2561965
Mouse α-human HIV-1 core antigen FITC (clone KC57)	Beckman Coulter	Cat#6604665
Mouse α-human p38 MAPK PE (clone 36/p38)	BD Biosciences	Cat#612565; RRID:AB_399856
Mouse α-human PLC-γ2 PE (clone K86-689.37)	BD Biosciences	Cat#558490; RRID:AB_647226
Mouse α-human Syk PE (clone I120-722)	BD Biosciences	Cat#558529, RRID:AB_647247
Mouse α-human Akt PE (clone M89-61)	BD Biosciences	Cat#560378; RRID:AB_1645328
Mouse IgG1 Isotype Control (clone 15H6)	AdipoGen	Cat#AG-35B-0003PF
Mouse IgG1, κ Isotype Ctrl (clone MOPC-21)	BioLegend	Cat#400153
α-goat IgG PE (polyclonal donkey IgG)	R&D Systems	Cat#F0107; RRID:AB_573123
Human Gamma Globulin	ThermoFisher Scientific	Cat#31879; RRID:AB_2532171
Oligonucleotides and other sequence-based reagents		
gRNA 5'ATAGTCCTGTCCATATTTGC3'	GenScript	Cat# SC1678
gRNA 5'GCGGGGAGGATTGAACCACG3'	GenScript	Cat# SC1678
Chemicals, Enzymes and other reagents		
BD CellFIX (10x concentrate)	BD Biosciences	Cat#340181; RRID: AB_2868724
BD Phosflow Fix Buffer I	BD Biosciences	Cat#557870; RRID: AB_2869102
BD Phosflow Perm Buffer III	BD Biosciences	Cat#558050; RRID: AB_2869118
Benzonase Nuclease, Purity >90%	Merck-Millipore	Cat#71205-3
Benzonase Nuclease, Purity > 99%	Merck-Millipore	Cat#70664-3
Blasticidin	InvivoGen	Cat#ant-bl-1
CellTracker Violet BMQC Dye	Invitrogen	Cat#C10094
CellTracker Orange CMTMR Dye	Invitrogen	Cat#C2927
CellTrace Far Red	Invitrogen	Cat#C34564
Immunocult Human CD3/CD28 T Cell Activator	Stemcell	Cat#10971; RRID:AB_2827806
Lenti-X Concentrator	Clontech Laboratories Inc.	Cat#631232
Lipofectamine 3000	Invitrogen	Cat#L3000-150
LIVE/DEAD Fixable Blue Dead Cell Stain Kit	Invitrogen	Cat#L34962
LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit	Invitrogen	Cat#L34976
Precision Count Beads	BioLegend	Cat#424902
Puromycin dihydrochloride	Sigma-Aldrich	Cat#P7255
Recombinant human DcR1 protein	Abcam	Cat#ab276266
Recombinant human DR4 protein	Abcam	Cat#ab641
Recombinant human DR5 protein	Abcam	Cat#ab243777
Recombinant human IL-2	PeproTech GmbH	Cat#200-02
Recombinant human IL-15	PeproTech GmbH	Cat#200-15
Recombinant human Osteoprotegerin protein	Abcam	Cat#ab182688
Streptavidin BV421	BioLegend	Cat#405226
Streptavidin PE	BioLegend	Cat#405204
Software		
Flowjo v10	BD Life Sciences	RRID:SCR_008520; https://www.flowjo.com/
GraphPad Prism 9	GraphPad Software	RRID:SCR_002798; https://www.graphpad.com/

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Reagents and Tools table (continued)

Reagent/Resource	Reference or Source	Identifier or Catalog Number
Other		
BD Cytofix/Cytoperm Fixation/Permeabilization Kit	BD Biosciences	Cat#554714; RRID:AB_2869008
DNeasy Blood and Tissue Kit	QIAGEN	Cat#69506
EasySep Human CD4 Positive Selection Kit II	Stemcell	Cat#17852
EasySep Human $CD4^+$ T Cell Enrichment Kit	Stemcell	Cat#19052
EasySep Human NK Cell Enrichment Kit	Stemcell	Cat#19055
Granzyme B Human ELISA Kit	ThermoFisher Scientific	Cat#BMS2027
IFN gamma Human ELISA Kit	ThermoFisher Scientific	Cat#EHIFNG
MACS Marker Screen, human	Miltenyi Biotec	Cat#130-110-055

Methods and Protocols

Materials availability

This study did not generate new unique reagents.

Experimental model and subject details

Human subjects

Peripheral blood was obtained from anonymized healthy human donors ($n = 71$) at the Institute for Transfusion Medicine, University Medical Center Hamburg-Eppendorf, Hamburg, Germany. Blood donors gave their general written consent to the usage of their blood samples for scientific studies in an anonymized form. The anonymized use of human material complies with a vote by the ethics committee of the German Medical Association. In addition, peripheral blood samples were obtained from healthy blood donors ($n = 27$) recruited at the University Medical Center Hamburg-Eppendorf, Hamburg, Germany. These donors provided written informed consent and studies were approved by the ethical committee of the Ärztekammer Hamburg (PV4780). Information on age and sex was not available for all subjects; however, was not relevant for the purpose of the study.

Cell lines

The HEK293T/17 cell line (ATCC, RRID:CVCL_1926) was used to generate infectious replication-competent HIV-1 virions. Cells were maintained in Dulbecco's modified Eagle medium, high-glucose, GlutaMAX Supplement, pyruvate (DMEM, Life Technologies) supplemented with 10% (v/v) heat-inactivated FBS (Biochrom) and 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich). The sex of the cell line is female.

Raji cells (RRID: CVCL_0511) were used to overexpress DR5. Therefore, the sequence of the receptor was obtained from GeneArt GeneSynthesis (Thermo Fisher) and cloned into a lentiviral transfer vector (pLVX-SIP) with puromycin resistance. Lipofectamine 3000 (Invitrogen) was used to transfect HEK239T/17 cells with the lentiviral transfer vector encoding the gene of interest, a VSV-G envelope vector (pCMV-VSV-G; Addgene) and a HIV-1 Gag-Pol packaging vector (psPAX2; NIH HIV Reagent Program). The supernatant containing the lentivirus was harvested 48 h after transfection and used for transduction of Raji cells. $DR5^{++}$ Raji cells were selected with 1 µg/ml puromycin (Sigma-Aldrich) 3 days post-transduction and later sorted for high DR5 expression with fluorescence-activated cell sorting. The generated cell line was maintained in complete medium (RPMI-1640 medium, Life Technologies) supplemented with 10% (v/v) heat-inactivated FBS (Biochrom), 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma–Aldrich), and 1 µg/ml puromycin (Sigma-Aldrich) and cultured at 37°C and 5% CO_2. The sex of the cell lines is male.

The cell line B-LCL 721.221 (.221) (RRID: CVCL_6263) (Shimizu & DeMars, 1989) was used to assess the function of NK cells. The cell line was maintained in complete medium [RPMI-1640 medium (Life Technologies) supplemented with 10% (v/v) heat-inactivated FBS (Biochrom), 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma-Aldrich)]. The sex of the cell line is female. 721.221 DR4/DR5 knockout cells were generated by CRISPR/Cas9-based gene targeting. First, 721.221 cells were transduced with lentiviral particles containing plentiCas9-Blast, a lentiviral plasmid construct that delivers hSpCas9 and blasticidin resistance. The lentiviral particles required for this purpose were produced in HEK293/17 cells as described for the transduction of Raji cells. Three days post-transduction, $Cas9^+$ cells were selected with 5 µg/ml blasticidin (InvivoGen). In the next step, $Cas9^+$ 721.221 cells were transduced with lentivirus containing lentiGuide-Puro plasmid constructs encoding a puromycin resistance and either a specific gRNA sequence to knockout DR4 or DR5. These plasmid constructs were generated by GenScript Biotech. The gRNA sequences used were as follows: 5'GCGGGGAGGATTGAACCACG3' (knockout of DR4) and 5'ATAGTCCTGTCCATATTTGC3' (knockout of DR5). Lentiviral particles were produced by transfection of HEK293/17 cells as described above. Transduced cells were selected with 0.5 µg/ml puromycin (Sigma-Aldrich) 3 days post-transduction and were later sorted for the absence of DR4 and DR5 expression. The generated 721.221 DR4/DR5 knockout cell line was maintained in complete medium (RPMI-1640 medium, Life Technologies) supplemented with 10% (v/v) heat-inactivated FBS (Biochrom), 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma-Aldrich), 0.5 µg/ml puromycin (Sigma-Aldrich), and 5 µg/ml blasticidin (InvivoGen) and cultured at 37°C and 5% CO_2. Expression levels of DR4 and DR5 on untransduced and transduced cell lines are displayed in Fig EV2. All cell lines are regularly tested for mycoplasma contamination.

Method details

Sample processing

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation within a few hours after phlebotomy from peripheral blood of healthy human donors and washed before

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resuspension in complete medium (RPMI-1640 medium (Life Technologies) supplemented with 10% (v/v) heat-inactivated FBS (Biochrom), 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma-Aldrich)). PBMCs were directly used for experiments or cryopreserved in FBS supplemented with 10% (v/v) dimethyl sulfoxide (Sigma-Aldrich) and stored in liquid nitrogen for future experimental applications. Thawing of previously cryopreserved PBMCs was performed by drop-wise addition of complete medium supplemented with 25 U/ml Benzonase Nuclease (Merck Millipore Novagen), washing and resting of cells at 37°C, 5% (v/v) CO_2 for app. 4 h before further use.

Enrichment of CD4 T Cells and NK Cells

Primary human CD4 T cells and NK cells were enriched from PBMCs through negative-selection strategy using EasySep Human $CD4^+$ T Cell Enrichment Kit (Stemcell) and EasySep Human NK Cell Enrichment Kit (Stemcell), respectively. Isolated CD4 T cells and NK cells were washed and then resuspended in appropriate complete medium for downstream applications. Purity of the enriched cell populations was verified by flow cytometry. Purity of CD4 T cells was 96.8% ± 2.1 (Mean ± SD); purity of NK cells was 86.0% ± 11.8% (Mean ± SD).

Primary infectious molecular clones of HIV-1

The following reagents were obtained through the NIH HIV Reagent Program, Division of AIDS, NIAID, NIH: Human Immunodeficiency Virus 1 (HIV-1), Strain NL4-3 Infectious Molecular Clone (pNL4-3), ARP-2852, contributed by Dr. M. Martin (Adachi *et al*, 1986); Human Immunodeficiency Virus 1 (HIV-1), Strain JR-CSF Infectious Molecular Clone (pYK-JRCSF), ARP-2708, contributed by Dr. Irvin SY Chen and Dr. Yoshio Koyanagi. Plasmids harboring the proviral genome of infectious molecular clones representing the HIV-1 primary strains CH198 (Parrish *et al*, 2013) and CH236 (Fenton-May *et al*, 2013) were kindly provided by the Beatrice Hahn Laboratory (University of Pennsylvania). The primary transmitted founder HIV-1 clone WITO was inferred from plasma viral sequences as reported previously (Ochsenbauer *et al*, 2012) and kindly provided by Mary Carrington (National Cancer Institute). Infectious viral particles were produced by transfecting HEK293T/17 cells with the respective plasmids using Lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol. Cell culture supernatants were harvested through centrifugation 72–96 h after transfection and then sterile filtered through either PES-based 0.45 µl or 0.22 µl Filtropur (Sarstedt) or 0.45 µl Steriflip-PVDF filter (Merck-Millipore). Subsequently, Lenti-X Concentrator (Clontech Laboratories Inc.) was used to increase viral titers according to the manufacturer's protocol. Lentiviral particles were aliquoted in DMEM (Life Technologies) and stored at −80°C until further use.

Infection of autologous CD4 T cells

After the enrichment of primary CD4 T cells, cells were washed and resuspended in complete medium supplemented with 100 U/ml IL-2 (PeproTech). Cells were then stimulated for 3 days with αCD3/αCD28 antibodies (Stemcell) at a concentration of 25 µl/ml at 37°C, 5% (v/v) CO_2. *In vitro* infection with various HIV-1 strains was performed through spinoculation of cells (1,200 rcf, 2 h, 37°C) with infectious viral particles at an MOI between 0.005 and 0.01 (O'Doherty *et al*, 2000). After centrifugation, cells were incubated at 37°C, 5% (v/v) CO_2 for 72–96 h, at 3.5–4 × 10^6 cells/ml. Cell culture supernatant of HIV-1-infected CD4 T cells and Mock CD4 T cells was replaced with fresh complete medium supplemented with 100 U/ml IL-2 (PeproTech) after 48 h.

Enrichment of HIV-1-infected CD4 T cells

Enrichment of HIV-infected cells (HIV-1 p24 positive, CD4 negative) was conducted based on previously described protocols (Davis *et al*, 2011). In brief, HIV-1-exposed CD4 T cells were incubated with 25 U/ml Benzonase Nuclease (Merck Millipore Novagen) for 30 min at 37°C, 5% (v/v) CO_2, and then washed. Infected cells were subsequently enriched through depletion of CD4-expressing T cells using EasySep Human CD4 Positive Selection Kit II (Stemcell). CD4 (−) cells in the supernatant were collected and used for downstream applications.

High-throughput functional analysis and TRAIL expression in NK cell subsets

NK cells were enriched from previously cryopreserved PBMCs and cultured overnight in complete medium supplemented with 50 U/ml IL-2 and 5 ng/ml IL-15 (PeproTech) at 37°C, 5% (v/v) CO_2. The next day, NK cells were co-incubated with autologous enriched HIV-1-infected (NL4-3, MOI = 0.01) CD4(−) T cells in complete-medium supplemented with 50 U/ml IL-2 and 5 ng/ml IL-15 (PeproTech) at an effector:target ratio of 1:2 for 5 h at 37°C, 5% (v/v) CO_2, in the presence of αCD107a-BV510 (BioLegend, clone H4A3). Cells were washed and then incubated with LIVE/DEAD Fixable Blue Dead Cell Stain (Invitrogen) in staining buffer (Dulbecco's Phosphate Buffered Saline (DPBS, Sigma Aldrich) + 2% (v/v) FBS + 1 mM EDTA (Sigma Aldrich)). Cells from up to four donors were then barcoded using αCD45 (BioLegend, clone 2D1) conjugated to AF700 or BV605: donor A: none; donor B: AF700; donor C: BV605; and donor D: AF700 + BV605 (Akkaya *et al*, 2016). Multiplexed cells were additionally labeled with αCD3-PerCP-Cy5.5 (BioLegend, clone UCHT1), αCD14-PerCP-Cy5.5 (BioLegend, clone HCD14), αCD16-BV785 (BioLegend, clone 3G8), αCD19-PerCP-Cy5.5 (BioLegend, clone HIB19), αCD56-BUV395 (BD Biosciences, clone NCAM16.2), αCD57-PE-Dazzle594 (BioLegend, clone HNK-1), αKIR2DL2/L3/S2-BV711 (BD Biosciences, clone DX27), αKIR3DL1-BV421 (BioLegend, clone DX9), αKIR2DL1/KIR2DS5-FITC (R&D Systems, clone 143211), αNKG2A-PC7 (Beckman Coulter, clone Z199), and αNKG2C-PE (Miltenyi Biotec, clone REA205). Cells of each donor were washed, merged together, and then individually stained with pre-titrated APC-conjugated antibodies for up to 327 selected surface antigens (Table EV1) and additional controls present in the human MACS marker screen (Miltenyi Biotec, Germany) following the manufacturer's instructions. Cells were fixed using 1× CellFIX (BD Biosciences) and stored in staining buffer until flow cytometric data acquisition. FMO controls were used to define gates and assess relative frequency of cells positive for each marker.

For independent validation and further in-depth analyses of the results obtained at the MACS marker screen, the experimental setup was repeated with the following modifications: HIV-1 *in vitro* infection of CD4 T cells was performed at an MOI of 0.005. Culture conditions with no target cells and Mock CD4 T cells were added. Antibody labeling for multiplexing, identification of NK cell subsets, and assessment of CD107a expression were performed as described above. LIVE/DEAD Fixable Near-IR Dead Cell Stain (Invitrogen) was

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used for discrimination of viable and dead cells. Cells were not distributed for antibody labeling of the MACS marker screen but only labeled with αTRAIL-APC (Miltenyi Biotec, clone RIK-2.1). NK cell subsets subject to further analyses comprised CD56Dim, CD56Bright, KIR-educated, NKG2A-educated, and uneducated NK cells. Definition of the education status of NK cells was based on the expression of the inhibitory receptors KIR2DL1/L2/L3, KIR3DL1, and NKG2A and the underlying HLA class I genotype. KIR-educated cells were defined as expressing at least one self-inhibitory KIR (2DL1/L2/L3, 3DL1) and being negative for NKG2A and NKG2A-educated cells as expressing NKG2A but lacking self-inhibitory KIR, and uneducated cells as lacking self-inhibitory KIR and NKG2A altogether.

Assessment of expression levels of death and decoy receptors

Ninety-six hours post-infection with NL4-3, WITO, CH198, CH236, JR-CSF, or no virus (Mock) CD4 T cells were treated with 25 U/ml Benzonase Nuclease (Merck Millipore Novagen) for 30 min at 37°C, 5% (v/v) CO_2. Cells were washed and then incubated separately in the presence of αCD45 (BioLegend, clone 2D1) conjugated to different fluorochromes for multiplexing (Akkaya *et al*, 2016). Cells were washed again, the different virus conditions of each donor were merged and stained with LIVE/DEAD Fixable Near-IR Dead Cell Stain (Invitrogen) as well as αCD3 (BioLegend, clone UCHT1) and αCD4 (BD Biosciences, clone RPA-T4) at 4°C. Cells were washed and then stained for 30 min with αDR4-Biotin (Miltenyi Biotech, clone DJR1) and αDR5-Biotin (Miltenyi Biotech, clone DJR2-4). Cells were washed before incubation with 0.2 μg/ml Streptavidin-PE (BioLegend) for 20 min at 4°C. Intracellular staining of HIV gag was performed using BD Cytofix/Cytoperm Fixation/Permeabilization kit (BD Biosciences) and αp24-FITC (Beckman Coulter, clone KC57). Cells were fixed with 1× CellFIX (BD Biosciences) and stored at 4°C until flow cytometric data acquisition. For the assessment of decoy receptor expression, CD4 T cells were infected with NL4-3 or no virus (mock). Four days post-infection, cells were treated with 25 U/ml Benzonase Nuclease (Merck Millipore Novagen) for 30 min at 37°C, 5% (v/v) CO_2. Cells were washed and then incubated with LIVE/DEAD Fixable Near-IR Dead Cell Stain (Invitrogen), αCD3 (BioLegend, clone UCHT1), αCD4 (BD Biosciences, clone RPA-T4), and goat αDcR1(R&D Systems, polyclonal) at 4°C for 20 min. Cells were washed again and incubated with αgoat-IgG-PE (R&D Systems, polyclonal) for additional 20 min at 4°C. Cells were permeabilized and stained for intracellular HIV gag using BD Cytofix/Cytoperm Fixation/Permeabilization kit (BD Biosciences) and αp24-FITC (Beckman Coulter, clone KC57). Cells were fixed with 1× CellFIX (BD Biosciences) and stored at 4°C until flow cytometric data acquisition.

Antibody-mediated blocking of TRAIL

Blocking of TRAIL interactions was conducted using a soluble TRAIL antibody (BioLegend, clone RIK-2). For co-incubation and blocking experiments with 721.221, isolated NK cells were resuspended in complete medium supplemented with 100 U/ml IL-2 and 10 ng/ml IL-15 (PeproTech) and incubated for 3 days to induce TRAIL expression (Kayagaki *et al*, 1999b; Balzarolo *et al*, 2013). TRAIL expression was monitored by flow cytometry using αTRAIL-APC (Miltenyi Biotec, clone RIK-2.1). TRAIL expression after 3 days was 81.3% ± 8.6% (Mean ± SD). For TRAIL-blocking experiments, NK cells were resuspended at a concentration of 5×10^5 cells/ml and were pre-incubated with 20 μg/ml purified αTRAIL or 20 μg/ml purified IgG1, κ isotype control antibody (BioLegend, clone MOPC-21) in complete medium supplemented with 100 U/ml IL-2 and 10 ng/ml IL-15 for 30 min at 37°C, 5% (v/v) CO_2. For DR4/DR5-blocking experiments, 721.221 were pre-incubated with 20 μg/ml purified αDR4 (AdipoGen, clone HS101) and 20 μg/ml purified αDR5 (AdipoGen, clone HS201) or the same amount of purified IgG1 isotype control antibody (AdipoGen, clone 15H6) for 30 min at 37°C, 5% (v/v) CO_2. After pre-incubation NK cells and 721.221 were co-incubated at an effector:target ratio of 1:1 in the presence of αCD107a-BV510 (BioLegend, Clone H4A3) for 5 h at 37°C, 5% (v/v) CO_2. During co-incubation, the respective blocking antibodies also remained present (final concentration per blocking antibody: 10 μg/ml). At the end of the co-incubation, cells were spun down and supernatants were stored at −20°C for further analysis by ELISA. Cells were stained with the viability dye LIVE/DEAD Fixable Near-IR (Invitrogen), αCD3-PerCPCy5.5 (BioLegend, clone UCHT1), αCD14-PerCPCy5.5 (BioLegend, clone HCD14), αCD16-BV785 (BioLegend, clone 3G8), αCD19-PerCPCy5.5 (BioLegend, clone HIB19), and αCD56-BUV395 (BD Biosciences, NCAM16.2). Thereafter, cells were fixed with 1× CellFIX (BD Biosciences) and analyzed using a BD LSRFortessa (BD Biosciences).

For co-incubation with autologous HIV-1-infected (NL4-3) CD4 T cells, NK cells, isolated from cryopreserved PBMCs, were stimulated overnight in complete medium supplemented with 100 U/ml IL-2 and 10 ng/ml IL-15 (PeproTech) to induce TRAIL expression. This modification was necessary due to the different workflow to generate autologous HIV-1-infected CD4 T cells. TRAIL expression was monitored by flow cytometry using αTRAIL-APC (Miltenyi Biotec, clone RIK-2.1). TRAIL expression after overnight incubation was 69.8% ± 8.3% (Mean ± SD). According to the previous description, NK cells were resuspended at a concentration of 5×10^5 cells/ml and were pre-incubated with 20 μg/ml purified αTRAIL or 20 μg/ml purified IgG1, κ isotype control antibody for 30 min at 37°C, 5% (v/v) CO_2. Then, NK cells and HIV-1-infected CD4 T cells were co-incubated at an effector:target ratio of 1:2 in the presence of αCD107a-BV510 (BioLegend, Clone H4A3) for 5 h at 37°C, 5% (v/v) CO_2. During co-incubation, the respective blocking antibodies also remained present (final concentration per blocking antibody: 10 μg/ml). Subsequent antibody labeling and analysis were carried out as described above.

TRAIL cross-linking

Immobilized (plate-coated) antibodies and proteins were used to induce clustering of various NK cell receptors and subsequent degranulation. Isolated NK cells were washed, resuspended in complete medium supplemented with 100 U/ml IL-2 and 10 ng/ml IL-15 (PeproTech), and incubated for 3 days to induce TRAIL expression (Kayagaki *et al*, 1999b; Balzarolo *et al*, 2013). TRAIL expression was monitored by flow cytometry using αTRAIL-APC (Miltenyi Biotec, clone RIK-2.1). Mean TRAIL expression after 3 days was 87% ± 6.9% (Mean ± SD). Sterile non-tissue culture-treated flat-bottom 96-well plates (Corning) were coated with purified αTRAIL (BioLegend, clone RIK-2), purified αNKG2D (BioLegend, clone 1D11), purified αNKp46 (Miltenyi Biotec, clone 9E2), purified IgG1, κ Isotype control antibody (BioLegend, clone MOPC-21), human DR4 protein (Abcam), human DR5 protein (Abcam), or human IgG (ThermoFisher Scientific) diluted in PBS at three

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different concentrations (1, 5, and 10 μg/ml) or were left uncoated (PBS). Coated plates were incubated at 4°C for at least 24 h. Before use, plates were washed six times with PBS. Directly thereafter, isolated NK cells were distributed (2×10^4 cells/well) on coated plates and incubated for 5 h at 37°C, 5% (v/v) CO_2 in the presence of αCD107a-BV510 (BioLegend, Clone H4A3). After incubation, supernatants were collected and stored at −20°C for further analysis. Cells were stained with the viability dye LIVE/DEAD Fixable Near-IR (Invitrogen), αCD3-PerCP-Cy5.5 (BioLegend, clone UCHT1), αCD14-PerCP-Cy5.5 (BioLegend, clone HCD14), αCD16-BV785 (BioLegend, clone 3G8), αCD19-PerCP-Cy5.5 (BioLegend, clone HIB19), and αCD56-BUV395 (BD Biosciences, NCAM16.2). For analysis by flow cytometry, cells were subsequently fixed with 1× CellFIX (BD Biosciences). Acquisition was carried out using a BD LSRFortessa (BD Biosciences).

Conjugate assay

To assess the impact of TRAIL interactions on the ability of NK cells to attach to target cells, we performed a conjugate assay as previously described (Burshtyn *et al*, 2000). In brief, enriched primary NK cells were incubated for 3 days in complete medium supplemented with 100 U/ml IL-2 and 10 ng/ml IL-15 (PeproTech) to induce TRAIL expression. TRAIL expression was assessed by flow cytometry using αTRAIL-APC (Miltenyi Biotec, clone RIK-2.1). Effector and target cells were individually labeled with a viability dye (LIVE/DEAD Fixable Near-IR, Invitrogen) and fluorescent dyes (NK cells: 2 mM CellTracker Violet BMQC, Invitrogen; 721.221: 1 mM CellTracker Orange CMTMR, Invitrogen). After exclusion of dead cells, NK cells were identified as events positive for CellTracker Violet. Conjugates of NK cells and 721.221s were determined as events positive for both, CellTracker Violet and Orange. The relative frequency (%) of NK cells in conjugates was determined as events in conjugates in relation to the total number of NK cells.

Enzyme-linked immunosorbent assay (ELISA)

Release of granzyme B and the production of IFNγ in *in vitro* NK cell assays were determined through commercially available ELISA kits. The limit of detection was 0.2 pg/ml for the Granzyme B ELISA kit (Invitrogen) and 2 pg/ml for the Human IFNγ ELISA kit (Invitrogen), respectively.

Phospho-Epitope staining

Immobilized (plate-coated) antibodies were used to induce clustering of various NK cell receptors and subsequent signaling cascades. Enriched NK cells were cultured in complete medium supplemented with 100 U/ml IL-2 and 10 ng/ml IL-15 (PeproTech) for 4 days to induce TRAIL expression. Sterile non-tissue culture-treated flat-bottom 96-well plates (Corning) were coated with either purified αTRAIL (BioLegend, clone RIK-2), purified αNKG2D (BioLegend, clone 1D11), purified αNKp46 (Miltenyi Biotec, clone 9E2), and purified IgG_1 κ Isotype control antibody (BioLegend, clone MOPC-21), diluted in PBS at 10 μg/ml or were left uncoated (PBS). Coated plates were incubated at 4°C for at least 24 h. Before use, plates were washed six times with PBS. Directly thereafter, isolated NK cells were distributed (1×10^5 cells/well) on coated plates, and incubated for 30 min at 37°C, 5% (v/v) CO_2. Cells were immediately fixed in BD Phosflow Fix Buffer I (BD Biosciences) for 10 min at 37°C, washed, and then permeabilized by incubating cells in BD Phosflow Perm Buffer III (BD Biosciences) for 30 min on ice. Cells were then washed and individually labeled with the following phospho-epitope-specific antibodies: PE mouse αPLC-γ2 (pY759), PE mouse αSyk (pY348), PE mouse αp38 MAPK (pT180/pY182), and PE mouse αAkt (pS473) (all BD Biosciences). Afterwards, cells were washed and fixed in 1× CellFIX (BD Biosciences). Acquisition of cells was carried out using a BD LSRFortessa (BD Biosciences).

Cytotoxicity assays

Three cytotoxicity assays were performed to confirm the impact of TRAIL on NK-cell-mediated cytotoxicity. Enriched NK cells were cultured in complete medium supplemented with 100 U/ml IL-2 and 10 ng/ml IL-15 (PeproTech) for 3 days to induce TRAIL expression. 721.221 cells (DR4/5 positive), .221-Cas9 (DR4/5 positive), and Raji-$DR5^{++}$ (overexpression of DR5) were used as target cells; and .221-DR4/5KO (DR4/5 knockout) and Raji-pSIP (baseline expression of DR5) cells served as control cells for their respective counterparts. Effector, control, and target cells were individually labeled with the following fluorescent dyes: CellTracker Violet BMQC, CellTracker Orange CMTMR, and Celltrace Far Red (all Invitrogen). TRAIL blocking condition: 1×10^5 NK cells were pretreated with either αTRAIL or isotype control (20 μg/ml) for 30 min and then co-cultured with 721.221 cells at an E:T ratio of 1:1. DR4/5KO condition: 1×10^5 NK cells were co-cultured with .221-DR4/5-KO and .221-Cas9 cells at an E:C:T ratio of 1:0.5:0.5. DR5 overexpression condition: 1×10^5 NK cells were co-cultured with Raji-pSIP and Raji-$DR5^{++}$ at an E:C:T ratio of 1:0.5:0.5. Conditions without the addition of NK cells served as references ("No NK" control). Cell suspensions were cultured for 5 h in 5-ml Polystyrene round-bottom tubes (Corning) at a final volume of 200 μl at 37°C, 5% (v/v) CO_2. After incubation, 25 μl of precision counting beads (BioLegend) were added and cells were immediately counted at a BD LSRFortessa (BD Biosciences). Data analysis: Cell counts were normalized to 10,000 counting beads. For the TRAIL blocking condition, relative frequency of remaining cells compared to the "No NK" control was used as a readout. For the DR5 overexpression and DR4/5KO conditions, specific lysis of designated target cells was calculated as previously described (Stary *et al*, 2020) with the following equation: $[1 - (\#\text{control cells}/\#\text{target cells})_{\text{no NK cells}}/(\#\text{control cells}/\#\text{target cells})_{\text{with NK cells}}] \times 100$.

HLA class I genotyping

DNA was extracted from previously cryopreserved PBMCs using DNeasy Blood and Tissue kit (QIAGEN). Samples were sent to DKMS Life Science Lab (Dresden, Germany) where genotyping for HLA class I alleles was performed. Genotypic data of donors displayed in Fig 2 are stated in Table EV2.

Data acquisition and statistical analysis

Acquisition of flow cytometry data was performed on a BD LSRFortessa (BD Biosciences) and further analyzed using FlowJo software v10.7 (FlowJo, LLC, BD Life Sciences). Quantification of granzyme B and IFNγ through ELISAs was performed on a Safire2 microplate reader (Tecan Austria, GmbH). Statistical analyses and graphical display of the data were conducted using GraphPad Prism, version 9 (GraphPad Software, La Jolla, CA, USA). Data of samples, for which processing and technical errors could not be excluded, as well as data of cell populations (% of positive cells), for which the parent

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population (denominator of the %) was < 50 cells, were set as missing, and analyses performed on available (non-missing) data. Hence, the total number of individuals analyzed in the high-throughput screen ($n = 19$) might not represent the number of individuals analyzed for each single of the 327 surface markers investigated (ranging from $n = 13$ to $n = 19$). Non-parametric statistical tests were applied to test for differences between groups. Wilcoxon signed-rank test was used for two groups with paired values. For the MACS marker screen, paired comparisons of $CD107a^+$ and $CD107a^-$ NK cell subsets were adjusted for multiplicity using the Benjamini and Hochberg false discovery rate (FDR) (Benjamini & Hochberg, 1995). Differential expression was assumed if a median intra-donor difference of > 5% p.p. between $CD107a^+$ and $CD107a^-$ NK cells and simultaneously FDR-adjusted P-values < 0.05 were present. For all other subsequent specific analyses, Bonferroni correction was used and applied to comparisons of interest. Spearman's ρ was used to test for association between two parameters. Calculation of HIV-I-specific responses was carried out as follows: HIV-I-specific response = [%$CD107a^+$ NK cells (HIV) − %$CD107a^+$ NK cells (Mock)]/[100 − %$CD107a^+$ NK cells (Mock)] * 100. Calculation of relative fluorescence intensity (RFI) was conducted as follows: RFI = [MdFI DR4/5/MdFI secondary ab control] − 1. Statistical parameters are stated in the results section as well as in the figure legends.

Data availability

This study includes no data deposited in external repositories. Raw data storage is performed by the Leibniz Institute of Virology on an internal server. Raw data will be made available upon request and can be shared after confirming that data will be used within the scope of the originally provided informed consent.

Expanded View for this article is available online.

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Author contributions
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Disclosure and competing interests statement
The authors declare that they have no conflict of interest.

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Expanded View Figures

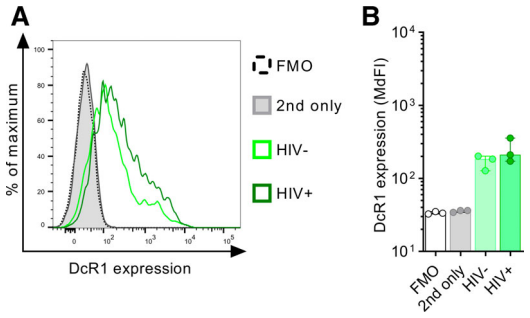

Figure EV1. DcR1 is expressed on CD4 T cells.

Primary enriched CD4 T cells were stimulated for 3 days and then infected with the HIV strain NL4-3. Four days post-infection cells were labeled with LIVE/DEAD Fixable Near-IR Stain, followed by incubation with αCD4-APC, αp24-FITC, goat anti-human DcR1, and then labeled with anti-goat-PE. Expression was measured as median fluorescence intensity (MdFI) by flow cytometry.

A Representative flow cytometry histogram of DcR1 expression on uninfected (HIV$^-$: CD4$^+$/p24$^-$) and infected (HIV$^+$: CD4$^-$/p24$^+$) cells in comparison to the anti-goat-PE control (grey) or FMO control (dashed line).

B Cumulative data of DcR1 expression displayed as MdFI. Data points represent the mean of two technical replicates per condition of three different donors (n = 3). Bar graphs show the median. Error bars show the IQR.

Source data are available online for this figure.

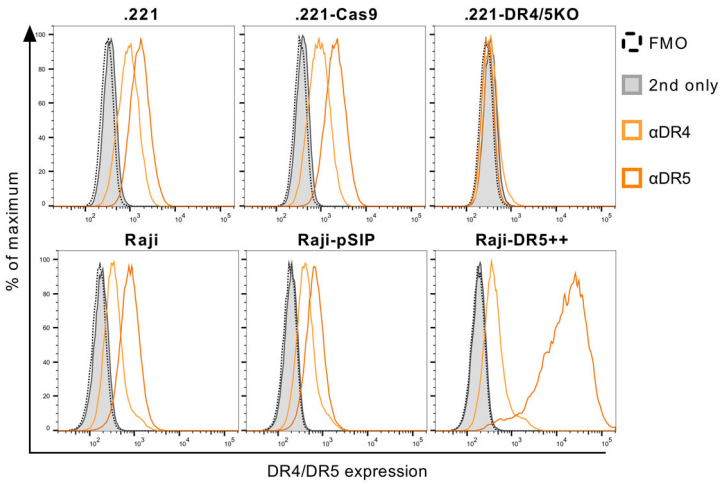

Figure EV2. Expression of TRAIL receptors on transduced 721.221 and Raji cells.

The expression of DR4 and DR5 was assessed by flow cytometry. 721.221 and Raji cells were labeled with LIVE/DEAD Fixable Near-IR Stain, followed by incubation with biotin-conjugated mouse anti-human DR4 or DR5, and then labeled with Streptavidin-BV421. Expression was quantified as fluorescence intensity. Representative histogram of DR4 (light orange) and DR5 (dark orange) expression in comparison to the Streptavidin-only control (grey) or the FMO control (dashed line). Upper panel (from left to right): untransduced 721.221 cells, Cas9-transduced .221s, and DR4/5 double knockout .221s. Lower panel (from left to right): untransduced Raji cells, Raji cells transduced with an empty vector (pSIP), and Raji cells overexpressing DR5.

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Table EV2: HLA class I genotypes of selected donors

Donor ID	HLA-A		HLA-B		HLA-C		HLA-C group	Bw4/Bw6*
	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2	haplotype	haplotype
BC191107	01:01	01:01	08:01	57:01	06:02	07:01	1/2	4
BC191128A	24:02	26:01	15:01	57:01	03:03	06:02	1/2	4
BC191128B	02:01	11:01	15:01	44:02	03:03	05:01	1/2	4
BC191128C	03:01	24:02	07:02	07:02	07:CENAH	16:01	1/1	6/6
BC191128D	03:01	24:02	07:02	55:01	03:CEJXD	07:CENAH	1/1	6/6
BC200109A	25:01	68:01	07:02	18:01	07:CENAH	12:03	1/1	6/6
BC200109B	01:01	02:01	44:02	52:01	07:04	12:02	1/1	4
BC200109C	01:01	03:01	08:01	15:01	03:03	07:01	1/1	6/6
BC200109D	02:01	02:01	44:02	51:07	05:01	14:02	1/2	4
BC200130A	02:01	29:02	44:03	55:01	03:03	16:01	1/1	4
BC200130B	02:01	31:01	15:01	40:01	03:CEJXD	03:CEJXD	1/1	6/6
BC200130C	02:01	02:01	13:02	49:01	06:02	07:01	1/2	4
BC200130D	01:01	02:01	15:01	57:01	03:CEJXD	06:02	1/2	4

*Bw6: Both HLA-B alleles encode for alleles with a Bw6 epitope motif. Bw4: At least one allele carries a Bw4 epitope motif.

Table EV 1: Analysed antigens of human MACS marker screen

No.	Antigen	No.	Antigen	No.	Antigen	No.	Antigen	No.	Antigen
1	CD1a	67	CD64	133	CD143 (ACE)	199	CD238 (KEL)	265	CLA
2	CD1b	68	CD66acde	134	CD144 (VE-Cadherin)	200	CD239 (BCAM)	266	CLEC2A
3	CD1c	69	CD66b	135	CD146	201	CD240DCE	267	CLEC12A
4	CD1d	70	CD66c	136	CD147	202	CD243 (ABCB1)	268	CLIP
5	CD2	71	CD68	137	CD148	203	CD244 (2B4)	269	CX3CR1
6	CD4	72	CD69	138	CD150 (SLAM)	204	CD246 (ALK)	270	DCIR
7	CD5	73	CD70	139	CD151	205	CD253	271	DLL1
8	CD6	74	CD71	140	CD152	206	CD256 (APRIL)	272	DLL4
9	CD7	75	CD72	141	CD154	207	CD258 (LIGHT)	273	DR3
10	CD8	76	CD73	142	CD155	208	CD262	274	ErbB-3 (Her3)
11	CD9	77	CD74	143	CD156a (ADAM8)	209	CD263 (TRAIL-R3)	275	FceRIa
12	CD10	78	CD79a	144	CD156c (ADAM10)	210	CD266 (FN14)	276	Fibroblast
13	CD11a	79	CD79b	145	CD157 (BST-1)	211	CD268	277	fMLP receptor
14	CD11b	80	CD80	146	CD161	212	CD269 (BCMA)	278	Galectin-3
15	CD11c	81	CD81	147	CD162	213	CD270 (HVEM)	279	Galectin-9
16	CD13	82	CD82	148	CD163	214	CD271 (LNGFR)	280	GARP (LRRC32)
17	CD15	83	CD83	149	CD164	215	CD273 (PD-L2)	281	GITR
18	CD18	84	CD84	150	CD165	216	CD275 (B7-H2)	282	GLAST (ACSA-1)
19	CD20	85	CD85a (ILT5)	151	CD166	217	CD276	283	GPR56
20	CD21	86	CD85d (ILT4)	152	CD169 (Siglec-1)	218	CD277	284	HLA-DM
21	CD22	87	CD85g (ILT7)	153	CD170 (Siglec-5)	219	CD278 (ICOS)	285	HLA-DQ
22	CD23	88	CD85h (ILT1)	154	CD171 (L1CAM)	220	CD279 (PD1)	286	HLA-DR
23	CD24	89	CD85j (ILT2)	155	CD172a (SIRPα)	221	CD282 (TLR2)	287	HLA-DR_DP_DQ
24	CD25	90	CD85k (ILT3)	156	CD172b (SIRPβ)	222	CD283	288	HLA-E
25	CD26	91	CD86	157	CD172g (SIRPγ)	223	CD284	289	IFNAR2
26	CD27	92	CD87	158	CD177	224	CD286 (TLR6)	290	IL-12R_b2
27	CD28	93	CD88 (C5AR)	159	CD178	225	CD295 (LEPR)	291	IL-17RC
28	CD29	94	CD89	160	CD180 (RP105)	226	CD298	292	IL-1RAcP
29	CD30	95	CD90	161	CD181 (CXCR1)	227	CD300e (IREM-2)	293	iNKT
30	CD31	96	CD93	162	CD182 (CXCR2)	228	CD300f (IREM1)	294	Integrin_b7
31	CD32	97	CD94	163	CD183 (CXCR3)	229	CD302 (CLEC13a)	295	Jagged2
32	CD33	98	CD95	164	CD184 (CXCR4)	230	CD303 (BDCA-2)	296	KLRG1 (MAFA)
33	CD34	99	CD96 (TACTILE)	165	CD185 (CXCR5)	231	CD304 (BDCA_4)	297	LAP (TGF-b1)
34	CD35	100	CD97	166	CD191 (CCR1)	232	CD305 (LAIR-1)	298	LGR5
35	CD36	101	CD98	167	CD192 (CCR2)	233	CD307a (FcRL1)	299	LT-bR
36	CD37	102	CD99	168	CD193 (CCR3)	234	CD307b (FcRL2)	300	Melanoma (MCSP)
37	CD38	103	CD100	169	CD194 (CCR4)	235	CD307e (FcRL5)	301	MICA/MICB
38	CD39	104	CD101	170	CD195 (CCR5)	236	CD309 (VEGFR-2/KDR)	302	MSCA-1 (W8B2)
39	CD40	105	CD103	171	CD196 (CCR6)	237	CD310 (VEGF-R3)	303	NKp80
40	CD41a	106	CD104 (Integrin b4)	172	CD197 (CCR7)	238	CD312 (EMR2)	304	Notch1
41	CD41b	107	CD105	173	CD199 (CCR9)	239	CD314 (NKG2D)	305	Notch2
42	CD42a	108	CD106	174	CD200	240	CD317 (BST2)	306	Notch3
43	CD42b	109	CD107b	175	CD201 (EPCR)	241	CD318 (CDCP1)	307	O4
44	CD43	110	CD110	176	CD202b (TIE-2)	242	CD319 (CRACC)	308	OSCAR
45	CD44	111	CD111	177	CD203c	243	CD324 (E-Cadherin)	309	Perforin
46	CD46	112	CD114	178	CD204	244	CD326 (EpCAM)	310	Plexin-D1
47	CD47	113	CD116	179	CD205	245	CD328 (Siglec-7)	311	PSA-NCAM
48	CD48	114	CD117 (A3C6E2)	180	CD206	246	CD329 (Siglec-9)	312	PSMA
49	CD49a	115	CD119	181	CD207 (Langerin)	247	CD335 (NKp46)	313	PTK7 (CCK-4)
50	CD49b	116	CD120a	182	CD208 (DC-LAMP)	248	CD336 (NKp44)	314	ROR1
51	CD49c	117	CD122 (IL-2Rβ)	183	CD209 (DC-SIGN)	249	CD337 (NKp30)	315	Siglec-10
52	CD49d	118	CD123	184	CD212	250	CD338 (ABCG2)	316	Siglec-5/Siglec-14
53	CD49e	119	CD126 (IL-6RA)	185	CD213a2 (IL-13Rα2)	251	CD340 (ErbB-2)	317	Siglec-8
54	CD49f	120	CD127	186	CD217	252	CD344 (Frizzled-4)	318	Slan (M-DC8)
55	CD51	121	CD131	187	CD218 (IL-18Rα)	253	CD352 (NTB-A)	319	SSEA-1
56	CD51/61	122	CD132	188	CD221 (IGF-1R)	254	CD353 (SLAMF8)	320	SSEA-4
57	CD52	123	CD133/1 (AC133)	189	CD222	255	CD354 (TREM-1)	321	SSEA-5
58	CD53	124	CD133/2 (293C3)	190	CD223	256	CRTAM	322	TdT
59	CD54 (ICAM-1)	125	CD134 (OX40)	191	CD226 (DNAM-1)	257	CD360 (IL-21R)	323	TIM-1
60	CD55 (DAF)	126	CD135	192	CD227 (Muc-1)	258	CD362 (Syndecan-2)	324	TIM-3
61	CD58 (LFA-3)	127	CD137	193	CD229 (Ly9)	259	A2B5	325	TLT-2
62	CD61	128	CD137L (4-1BBL)	194	CD230 (PrP)	260	Podoplanin	326	TRA-1-85 (CD147)
63	CD62E	129	CD138	195	CD231 (TALLA)	261	C3a receptor	327	TSPAN8
64	CD62L	130	CD140b	196	CD233	262	CCL2 (MCP-1)		
65	CD62P	131	CD141 (BDCA-3)	197	CD234 (DARC)	263	CCR10		
66	CD63	132	CD142	198	CD235a (Glycophorin A)	264	ChemR23 (CMKLR1)		

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Host KIR/HLA-C Genotypes Determine HIV-Mediated Changes of the NK Cell Repertoire and Are Associated With Vpu Sequence Variations Impacting Downmodulation of HLA-C

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NK cells play a pivotal role in viral immunity, utilizing a large array of activating and inhibitory receptors to identify and eliminate virus-infected cells. Killer-cell immunoglobulin-like receptors (KIRs) represent a highly polymorphic receptor family, regulating NK cell activity and determining the ability to recognize target cells. Human leukocyte antigen (HLA) class I molecules serve as the primary ligand for KIRs. Herein, HLA-C stands out as being the dominant ligand for the majority of KIRs. Accumulating evidence indicated that interactions between HLA-C and its inhibitory KIR2DL receptors (KIR2DL1/L2/L3) can drive HIV-1-mediated immune evasion and thus may contribute to the intrinsic control of HIV-1 infection. Of particular interest in this context is the recent observation that HIV-1 is able to adapt to host *HLA-C* genotypes through Vpu-mediated downmodulation of HLA-C. However, our understanding of the complex interplay between *KIR/HLA*

immunogenetics, NK cell-mediated immune pressure and HIV-1 immune escape is still limited. Therefore, we investigated the impact of specific *KIR/HLA-C* combinations on the NK cell receptor repertoire and HIV-1 Vpu protein sequence variations of 122 viremic, untreated HIV-1$^+$ individuals. Compared to 60 HIV-1$^-$ controls, HIV-1 infection was associated with significant changes within the NK cell receptor repertoire, including reduced percentages of NK cells expressing NKG2A, CD8, and KIR2DS4. In contrast, the NKG2C$^+$ and KIR3DL2$^+$ NK cell sub-populations from HIV-1$^+$ individuals was enlarged compared to HIV-1$^-$ controls. Stratification along *KIR/HLA-C* genotypes revealed a genotype-dependent expansion of KIR2DL1$^+$ NK cells that was ultimately associated with increased binding affinities between KIR2DL1 and HLA-C allotypes. Lastly, our data hinted to a preferential selection of Vpu sequence variants that were associated with HLA-C downmodulation in individuals with high KIR2DL/HLA-C binding affinities. Altogether, our study provides evidence that HIV-1-associated changes in the KIR repertoire of NK cells are to some extent predetermined by host *KIR2DL/HLA-C* genotypes. Furthermore, analysis of Vpu sequence polymorphisms indicates that differential KIR2DL/HLA-C binding affinities may serve as an additional mechanism how host genetics impact immune evasion by HIV-1.

Keywords: NK cell, KIR, HLA-C, HIV-1, Vpu

INTRODUCTION

Natural killer (NK) cells are innate lymphocytes crucially involved in antiviral immunity and tumor surveillance (1, 2). Their main effector functions comprise production of pro-inflammatory cytokines and elimination of virus-infected and transformed cells through direct cytotoxicity (3, 4). NK cells utilize a large number of activating and inhibitory receptors to distinguish between healthy ("self") and aberrant cells ("non-self") (5). Inhibitory receptors mainly interact with various human leukocyte antigen (HLA) class I molecules, maintaining self-tolerance, whereas receptors with activating properties are able to recognize stress ligands on potential target cells. An important group of NK cell receptors are killer-cell immunoglobulin-like receptors (KIRs) that predominantly recognize HLA class I molecules. The most recently evolved HLA class I molecule is HLA-C, which is only present in humans and great apes (6). Contrary to HLA-A and HLA-B, virtually all HLA-C allotypes are recognized by KIRs, making HLA-C a dominant ligand for the regulation of NK cell activity (7, 8). Co-evolution of HLA class I and KIRs resulted in a remarkable diversity with distinct binding specificities between their members (8, 9). The inhibitory receptor KIR3DL2 binds only to HLA-A3/11 (10, 11), while KIR3DL1 recognition is limited to a subset of HLA-A and -B molecules that contain the serological motif Bw4 (12). In contrast, HLA-C is recognized by a number of inhibitory and activating KIRs. HLA-C molecules can be distinguished into two groups, defined by a dimorphism at position 80 in the α1 domain (7). HLA-C group 1 (HLA-C1) allotypes are characterized by an asparagine at position 80 and are predominantly recognized by the inhibitory receptors KIR2DL2 and KIR2DL3 as well as some allotypes of the activating receptor KIR2DS2 (13). *HLA-C* alleles encoding for group 2 (HLA-C2) molecules contain a lysine at position 80 and interact with the inhibitory KIR2DL1, the activating KIR2DS1 and a few KIR2DS5 allotypes (14–16). Additionally, certain KIR2DL2 and KIR2DL3 allotypes are cross-reactive with HLA-C2 molecules. Finally, KIR2DS4 is able to bind HLA-C1 and -C2 molecules with variegated affinities (17, 18).

Both, HLA-C and KIRs, are highly polymorphic, resulting in a large number of allotype combinations within a population that are further characterized by variegating binding affinities (8, 19). Accumulating evidence strongly indicates that interactions between KIRs and HLA-C impact the course of pathologic conditions and infectious diseases, such as preeclampsia (20), arthritis (21) and HCV infection (22). Most prominently, several studies have demonstrated a major impact of *HLA class I* genes in the outcome of HIV-1 infection, alone or in combination with certain *KIR*s (23–27). In recent years accumulating evidence suggests a role for HLA-C in immune control as well as HIV-1-associated immune escape. For one, increased HLA-C expression levels were associated with protection against HIV-1 progression (28). The ability of the accessory HIV-1 protein Vpu to downregulate HLA-C expression level on infected cells (29) and its adaption to *HLA-C* genotypes through sequence variations in Vpu was indicative of a novel HLA-C-associated immune evasion strategy (30). Further epidemiological and experimental evidence demonstrated that KIR$^+$ NK cells can recognize HIV-1-mediated alterations of HLA-C expression (31) or changes in the HLA-C/peptide complex (32–37). However, the complex interplay between host genetics, NK cell-mediated immune pressure and HIV-1 immune escape is still only partially understood. In particular, the high allelic diversity of HLA-C and KIR2DL molecules and previous limitations in the resolution of

KIR alleles have complicated the generation of models that would allow a clearer view of the mechanisms underlying the intrinsic control of HIV-1 and the contribution of KIR2DL and their HLA-C ligands.

In this study, we investigated the impact of KIR/HLA-C interactions on the NK cell repertoire and HIV-1 sequence polymorphisms in the context of HIV-1 infection. For this, we determined the binding affinities of various KIR2DL/HLA-C allotype combinations and conducted a comprehensive phenotypical characterization of NK cells from a cohort of viremic, untreated HIV-1$^+$ individuals compared to HIV-1$^-$ controls. Finally, we performed next-generation sequencing (NGS) of *Vpu* from matched plasma and PBMC samples from HIV-1$^+$ individuals. Herein, our results indicated that the underlying host genetics influences the NK cell receptor repertoire in HIV-1$^+$ individuals as well as the selection of Vpu sequence variants.

MATERIALS AND METHODS

Human Subjects

Peripheral blood samples were obtained from healthy blood donors (n = 45) recruited at the University Medical Center Hamburg-Eppendorf, Hamburg, Germany. In addition, peripheral blood was obtained from anonymized healthy human donors (n = 15) at the Institute for Transfusion Medicine, University Medical Center Hamburg-Eppendorf, Hamburg, Germany. Information on age and sex was not available for all subjects, however was not relevant for the purpose of the study. Cryopreserved peripheral blood mononuclear cells (PBMC) and plasma samples from untreated HIV-1-infected individuals (n = 122) were obtained from the Translational Platform HIV (TP-HIV) Cohort by the German Center for Infection Research (DZIF).

Sample Processing

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood from healthy human donors by density gradient centrifugation. Isolated PBMCs were cryopreserved for upcoming experiments in liquid nitrogen tanks in heat-inactivated fetal bovine serum (Sigma-Aldrich) supplemented with 10% (v/v) DMSO (Sigma-Aldrich). Cryopreserved PBMCs were thawed by adding PBMCs dropwise to complete RPMI-1640 medium (Life Technologies) supplemented with 10% (v/v) heat-inactivated FBS (Sigma-Aldrich), 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma-Aldrich) and 25 U/ml Benzonase Nuclease (Merck Milipore Novagen). Thawed PBMCs were incubated for 30 min at 37°C and washed with PBS (Sigma-Aldrich) and then used for antibody staining and genomic DNA isolation.

Cell Lines

HEK293T/17 cell line (ATCC, Cat#CRL-11268) was cultured in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies) supplemented with 10% (v/v) heat-inactivated FBS (Sigma-Aldrich), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich) and used for the generation of lentivirus. Sf9 insect cell line (CVCL_0549) was used to produce high titer of baculovirus stocks cultured in Sf-900 II medium (Life Technologies) supplemented with 10% (v/v) heat-inactivated FBS, (fetal bovine serum Sigma-Aldrich), 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma-Aldrich) and 1% (v/v) L-glutamine (Life Technologies). Hi5 insect cell line (CVCL_C190) was used for KIR2DL-Fc fusion protein production and cultured in Express Five serum free medium (Life Technologies) supplemented with 1% (v/v) L-glutamine (Life Technologies). The HLA class I deficient B cell line 721.221 (RRID: CVCL_6263) (38) was used for expressing HLA-C1 and -C2 allotypes (**Table 1**). For this, the extracellular domains of HLA-C allotypes were obtained from GeneSynthesis (Thermo Fisher) and cloned into a lentiviral transfer vector (pSIP-ZsGreen) with a puromycin resistance. For the production of lentivirus, Lipofectamine 3000 (Life Technology) was used to transfect HEK293T/17 cells with the lentiviral transfer vector with the gene of interest, a VSV-G envelope vector (pHEF-VSVF; NIH HIV Reagent Program) and a HIV-1 Gag-Pol packaging vector (psPAX2; NIH HIV Reagent Program). After 48 h, the supernatant containing the lentivirus was harvested and used for the transduction of the 721.221 cells. 3 days post-transduction, HLA-C$^+$ 721.221 cells were selected with 1 µg/ml puromycin (Sigma-Aldrich) and later sorted for high HLA-C expression by fluorescence-activated cell sorting. HLA-C-721.221 cell lines were cultured in complete RPMI-1640 medium (Life Technology) supplemented with 10% (v/v) heat-inactivated FBS (Sigma-Aldrich), 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma-Aldrich) and 1 µg/ml puromcyin at 37°C and 5% CO_2.

Generation of KIR2DL-Fc Fusion Proteins

KIR2DL1*001-Fc, KIR2DL2*003-Fc and KIR2DL3*001-Fc fusion constructs were produced as described in Hilton et al. (39). Further KIR2DL-Fc fusion constructs (**Table 2**) were generated by site-directed mutagenesis (Agilent Technologies). KIR2DL-Fc fusion constructs were co-transfected with linearized baculovirus (Expression Systems) into Sf9 insect cells with Cellfectin II (Invitrogen). Sf9 cells were cultured for 7 days in Sf-900 II medium (Life Technologies) at 115 rpm, 27°C and 5% CO_2. To produce high titer of baculovirus, 3 more rounds of amplification were performed by culturing Sf9 cells in Sf-900 II medium supplemented with 10% (v/v) heat-inactivated FBS (Sigma-Aldrich), 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma-Aldrich) and 1% (v/v) L-glutamine (Life Technologies) for 4-5 days with the respective virus stock. Hi5 insect cells were

TABLE 1 | Overview of generated HLA-C expressing 721.221 cell lines.

Cell line	Allotypes
HLA-C1-721.221 cell lines	*01:02, *03:03, *03:04, *07:01, *07:02, *12:03, *14:02, *16:01
HLA-C2-721.221 cell lines	*02:02, *04:01, *05:01, *06:02

cultured in Express Five serum free medium (Life Technologies) supplemented with 1% (v/v) L-glutamine (Life Technologies). KIR2DL-Fc fusion proteins were produced by infecting Hi5 insect cells with P3 viral stock for 72 h at 115 rpm, 27°C and 5% CO_2. The supernatant was collected by centrifugation and filtration. To isolate the KIR2DL-Fc fusion proteins, the supernatant was neutralized with HEPES buffer (Thermo Fisher Scientific) and incubated with protein A Sepharose beads (Thermo Fisher Scientific) rotating overnight. The KIR2DL-Fc fusion proteins were washed with PBS and eluted with 100 mM glycine (pH 2.7) and immediately neutralized with 1 M Tris (pH 9). The eluted proteins were desalted by using a Sephadex G-25 desalting column (GE Healthycare). Protein concentration was measured with a BCA protein assay (Thermo Fisher Scientific).

KIR2DL-Fc Binding Assay

HLA-C-721.221 cell lines and untransduced 721.221 were incubated with 25 µg/ml KIR2DL-Fc fusion protein for 15 min at 4°C and then washed and stained with a LIVE/DEAD fixable Near-IR Dead Cell staining kit (Invitrogen) and with a secondary F(ab)2 goat anti-human IgG-PE antibody (Invitrogen). After additional washing steps, the cells were fixed in 1x CellFIX (BD Bioscience) and the binding of the KIR2DL-Fc fusion protein was analyzed *via* flow cytometry. As a negative control, cells were only stained with anti-human IgG-PE without any KIR2DL-Fc fusion protein. HLA-C expression of all transfected and untransfected 721.221 cell lines was assessed by flow cytometry using the HLA-ABC antibody W6/32 (**Supplementary Figure 1**). Binding of KIR2DL-Fc fusion proteins was normalized to the negative control and adjusted for the HLA-ABC expression of the respective 721.221 cell lines.

Phenotypical Characterization of PBMCs From Healthy and HIV-1-Infected Individuals

PBMCs from healthy and untreated HIV-1-infected individuals were gently thawed by adding dropwise complete medium supplemented with 25 U/ml Benzonase Nuclease (Merck Milipore Novagen) followed by a 30 min incubation at 37°C and 5% CO_2. After counting and washing with PBS, cells were incubated with LIVE/DEAD fixable Near-IR Dead Cell staining kit (Invitrogen) and the following antibodies at 4°C: anti-CD3-PerCP-Cy5.5 (clone UCHT1, BioLegend), anti-CD4-BV650 (clone RPA-T4, BioLegend), anti-CD8-AF700 (clone EB6B, BioLegend), anti-CD14-APC-Cy7 (clone HCD14, BioLegend), anti-CD19-APC-Cy7 (clone HIB19, BioLegend), anti-CD56-BUV395 (clone NCAM16.2, BD Optibuild), anti-CD16-BV785 (clone 3G8, BioLegend), anti-CD57-BV510 (clone QA17A04, BioLegend), anti-NKG2A-PE-Vio615 (clone REA110, Miltenyi), anti-NKG2C-BUV563 (clone 134591, BD Optibuild), anti-KIR2DL1/S1-APC (clone EB6B, Beckman Coulter), anti-KIR2DL1/S5-PE (clone 134211, R&D Systems), anti-KIR2DL2/L3/S2-BV711 (clone DX27, BD Optibuild), anti-KIR2DL3-AF488 (clone 180701, R&D Systems), anti-KIR3DL1-AF700 (clone DX9, BioLegend), anti-KIR3DL1/L2-PE-Vio770 (clone 5.133, Miltenyi) and anti-KIR2DS4-Biotin (clone JJC11.6, Miltenyi) with secondary Strepdavidin-BV421 (BioLegend). Cells were washed and then fixed with FluoroFix Buffer (BioLegend). Cells were analyzed by flow cytometry.

TABLE 2 | Overview of generated KIR2DL-Fc fusion proteins.

	Allotypes
KIR2DL1-Fc fusion proteins	*001, *003, *004, *020, *022
KIR2DL2-Fc fusion proteins	*001, *003, *009
KIR2DL3-Fc fusion proteins	*001, *002, *009, *016

The symbol "" represents a separator between the gene and the allele group. In this instance the allele group of the respective KIR2DL1, L2 and L3 proteins.*

Viral RNA Isolation, cDNA Synthesis and Genomic DNA Isolation

Viral RNA from plasma samples of HIV-1-infected individuals was isolated using the High Pure Viral RNA Kit (Roche) and then used for a reverse transcription reaction (SuperScript III One-Step RT-PCR, Invitrogen) with specific Vpu outer-revers primer (sense primer: 5'-CCT AGA CTA GAG CCC TGG AAG CAT-3', anti-sense primer: 5'-TTC TTG TGG GTT GGG GTC TGT-3') described by Pickering et al., 2014 (38). Genomic DNA from PBMCs was isolated using the DNeasy Blood & Tissue Kit (QIAGEN).

Vpu Sequencing

cDNA generated from viral RNA and genomic DNA isolated from PBMC of HIV-1-infected individuals was amplified and prepared for Vpu sequencing *via* PCR (Platinum SuperFi DNA Polymerase, Invitrogen) using gene specific Vpu inner-primers (38) and overhangs of Illumina-sequencing compatible adapter sequences (highlighted in bold) (sense primer: 5'-**TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG** TAA TAC GAC TCA CTA TAG GCA GGA AGA AGC GGA GAC A-3', anti-sense primer: 5′-**GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G**CA GGA AAC AGC TAT GAC CCC ATA ATA GAC TGT GAC-3′). In a second PCR using the KAPA HiFi HotStart ReadyMix PCR Kit (Roche) the Illumina-sequencing compatible adapter overhangs served as a template to add dual indices (Nextera XT Index Kit, Illumina) for multiplexing and to complete the Illumina sequencing adapters. Prior to sequencing samples were pooled according to their concentration measured *via* Qubit dsDNA High Sensitivity (Invitrogen). The amplicon size of each pool was assessed on a TapeStation 4200 High Sensitivity D1000 Screen Tape (Agilent Technologies) to dilute the samples to a concentration of 2 nM. Paired-end sequencing of the generated libraries was performed on an Illumina MiSeq platform (2x250 bp) aiming for 0.1 - 0.2 million reads per sample.

Vpu Sequence Analysis

Raw paired-end reads were aligned to indexed reference sequence AF324493.2 from NCBI GenBank using bwa mem command (40). Resulting SAM alignment files were converted to BAM files, sorted and indexed using samtools (41). Variants were detected using GATK HaplotypeCaller (42) using "–dont-use-soft-clipped-bases true" option, and to identify variant with

maximum frequency "–max-alternate-alleles 1" option was used. In downstream process low quality variants were filtered out using following parameters from GATK Best Practices recommendations: "DP < 25 || QD < 2.0 || MQ < 30.0 || FS > 60.0 || MQRankSum < -12.5 || ReadPosRankSum < -8.0" (43). Filtered high quality variants meeting the criteria were then taken as input to create mutation VPU nucleotide sequence taken 6061:6306 as reference coordinates using GATK FastaAlternateReferenceMaker command (44).

HLA Class I and KIR Genotyping

Genomic DNA was isolated from cryopreserved PBMCs with the DNeasy Blood and Tissue Kit (QIAGEN). HLA class I and KIR genotyping was performed by the DKMS Life Science Lab, Dresden, Germany as described in (45, 46).

Data Analysis and Statistics

The acquisition of flow cytometric data was performed with a BD LSRFortessa (BD Bioscience) in the core facility Fluorescence Cytometry at the Leibniz Institute of Virology and analyzed using FlowJo software 10.7.1 (BD Life Sciences). Graphical and statistical analyses were performed using GraphPad Prism 9.0.1 software (GraphPad Software, La Jolla, CA, USA). Multiple linear regression analysis was performed to assess the impact of HIV-1 status, age and sex on the expression of NK cell receptors (**Figure 2C**). One-way ANOVA, test for linear trend was used to test for gene-dose effects (Figure 2A, B, D, E). A non-parametric statistical test (Mann-Whitney) was applied to test for differences between two groups. Adjustment for multiplicity was applied to comparisons of interest using false discovery rate (FDR) with Q = 5% (Benjamini/Krieger/Yekutieli). Bonferroni correction was used in one instance (**Figures 3A, B**). Variation in the frequency of amino acid residues between individuals of different HLA-C genotypes was analyzed by chi square tests. Statistical parameters are stated in the results section as well as in the figure legends.

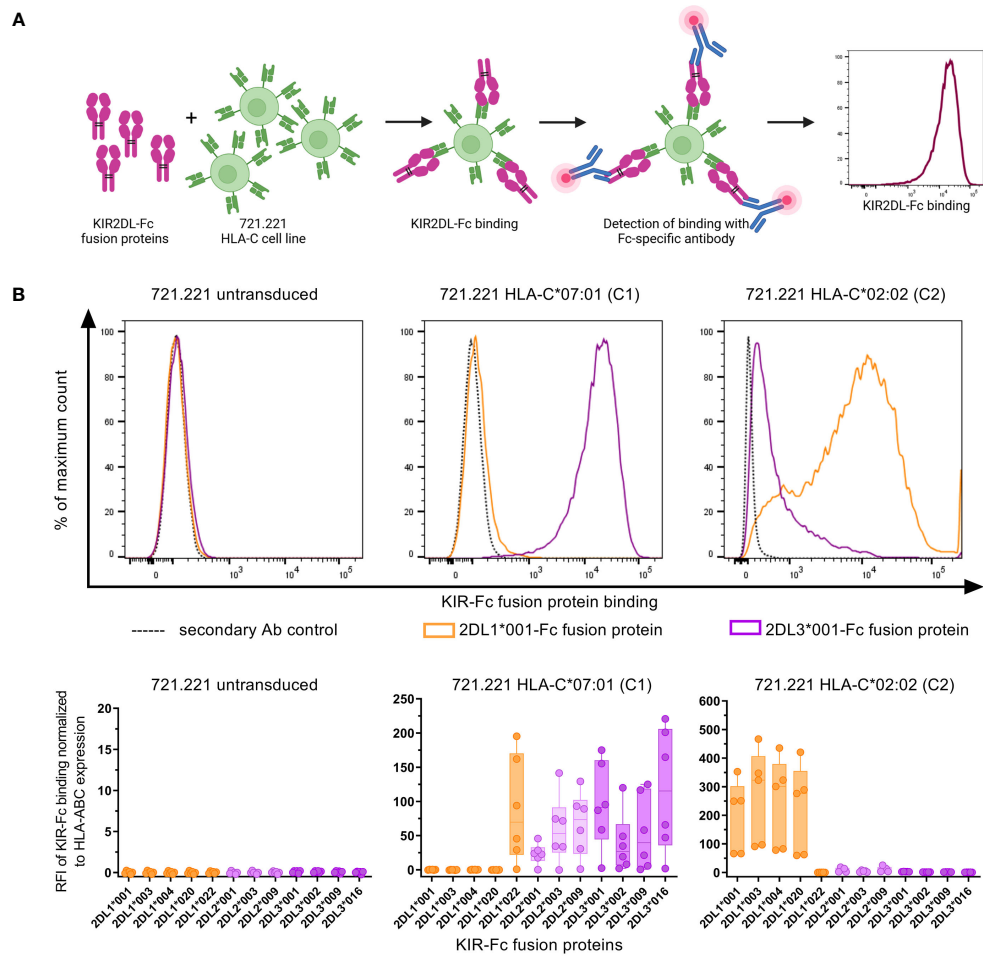
RESULTS

KIR2DL/HLA-C Allotype Combinations Display Differential Binding Affinities

Previous assessments of KIR binding affinities to HLA class I have repeatedly confirmed the overall specificities of KIRs to different groups of HLA class I molecules (7, 10, 12). Further stratification of HLA class I and KIR molecules into specific allotypes revealed that binding affinities are exhibited as a continuum, ranging from strong affinities to no binding at all (8, 47, 48). In order to assess the specificity and affinity of the receptors KIR2DL1, KIR2DL2, and KIR2DL3 (henceforth KIR2DL) to various HLA-C allotypes in a cellular system, we assessed binding of various KIR2DL-Fc fusion proteins to HLA-C expressing cell lines using flow cytometry (**Figure 1A**). Representative and cumulative data in **Figure 1B** display the overall binding specificities of the three KIR2DL types. All generated KIR2DL-Fc constructs did not bind to untransduced 721.221, while KIR2DL1-Fc allotypes were predominantly associated with binding of HLA-C group 2 (HLA-C2), but did not recognize HLA-C group 1 (HLA-C1) on 721.221s with the exception of KIR2DL1*022. KIR2DL1*022 is characterized by an amino acid substitution at position 44 which leads to a switch in its binding specificity towards HLA-C1 ligands (49). Conversely, KIR2DL2-Fc and KIR2DL3-Fc displayed binding affinities to both HLA-C1 and -C2. For comparison of the binding affinities of all tested KIR2DL/HLA-C allotype combinations, the highest value for KIR2DL-Fc binding was set to 100% and all other values were calculated accordingly (**Figure 1C**). The displayed affinity matrix indicated hierarchies that applied to KIR2DL1 as well as HLA-C2 allotypes. E.g. 2DL1*003 showed the highest affinities to HLA-C2 cell lines of all tested KIR2DL1 allotypes independent of the HLA-C allotype follwes by 2DL1*004, 2DL1*020 and 2DL1*001. In turn, highest KIR2DL1 binding was observed for the HLA-C2 cell line expressing C*05:01 irrespective of the KIR2DL1 allotype. This was followed by C*02:02, C*04:01 and C*06:02. Overall KIR2DL2-Fc and KIR2DL3-Fc constructs displayed a lower binding affinity to HLA-C expressing cell lines in general and showed a more ambiguous affinity pattern. E.g. the KIR2DL3*016/C*07:01 combination showed the strongest binding of all KIR2DL2/L3 allotypes to HLA-C1 cell lines. But for HLA-C2 cell lines KIR2DL2*009 showed the highest affinity, i.e. C*05:01. Of note, the HLA-C1 cell lines C*01:02, C*03:03, C*12:03 and C*14:02 did not allow for any KIR2DL-Fc binding in our experimental setting, whereas C*03:04, C*07:02 and C*16:01 demonstrated only low binding affinities to the KIR2DL2/L3-Fc fusion proteins. C*07:01 allowed for stronger binding of KIR2DL2/L3 allotypes than any other tested C1 cell line. Altogether, the performed KIR2DL-Fc fusion protein assays showed high binding affinities of KIR2DL1/HLA-C2 combinations but no binding of KIR2DL1 to HLA-C1. Furthermore, KIR2DL2/L3-Fc fusion proteins had only low binding affinities for both, HLA-C1 and -C2 expressing 721.221 cell lines.

HIV-1 Infection Is Associated With an Altered NK Cell Receptor Repertoire

Next, we investigated the impact of HIV-1 infection on the expression patterns of NK cell receptors. For this, we assessed the expression of eleven NK cell receptors within 60 HIV-1$^-$ and 122 untreated HIV-1$^+$ individuals (**Table 3**; **Figure 2A**). Exemplary expression patterns for all analyzed NK cell receptors and the applied gating are shown in **Figure 2B**. Initial comparative analyses of bulk NK cells, including adjustment for age and sex, suggested significant changes in the receptor repertoire between HIV-1$^+$ individuals and the HIV-1$^-$ control group (**Figure 2C** and **Supplementary Table 1**). We observed a lower percentage of NK cells expressing the inhibitory receptor NKG2A in HIV-1$^+$ individuals (median: 42.5%) compared to our control group (54.4%, $p < 0.0001$ *vs.* HIV-1-), whereas the percentage of NK cells expressing the activating counterpart NKG2C was considerably increased (23.5% *vs.* 3.5%, $p < 0.0001$ *vs.* HIV-1-). The relative frequency of NK cells expressing CD57,



C

721.221 HLA-C cell lines		KIR-Fc fusion proteins											
		2DL1 *003	2DL1 *004	2DL1 *020	2DL1 *001	2DL1 *022	2DL3 *016	2DL3 *001	2DL2 *009	2DL2 *003	2DL3 *009	2DL2 *001	2DL3 *002
	721.221	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0.1	0.1
HLA-C1	C*14:02	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.0	0.0	0.1	0.0	0.0
HLA-C1	C*12:03	0.0	0.0	0.0	0.0	0.1	0.1	0.2	0.2	0.0	0.1	0.0	0.1
HLA-C1	C*01:02	0.0	0.0	0.0	0.0	0.1	0.1	0.3	0.1	0.1	0.1	0.0	0.1
HLA-C1	C*03:03	0.0	0.0	0.0	0.0	0.2	0.2	0.3	0.3	0.1	0.1	0.0	0.1
HLA-C1	C*16:01	0.0	0.0	0.0	0.1	0.1	1.5	0.5	0.8	0.8	0.1	0.2	1.2
HLA-C1	C*03:04	0.0	0.0	0.0	0.0	0.3	0.5	0.9	0.8	0.4	0.1	0.2	0.1
HLA-C1	C*07:02	0.0	0.1	0.0	0.1	0.5	2.0	1.1	3.3	1.6	0.5	0.7	1.7
HLA-C1	C*07:01	0.0	0.1	0.0	0.1	18.9	31.3	24.8	19.9	14.4	10.8	6.3	7.3
HLA-C2	C*06:02	7.1	3.1	2.4	2.3	0.1	0.1	0.8	0.9	0.4	0.5	0.7	0.4
HLA-C2	C*04:01	59.4	47.7	40.0	31.4	0.1	0.1	0.4	0.7	0.4	0.2	0.5	0.2
HLA-C2	C*02:02	87.5	81.7	75.0	67.6	0.1	0.2	0.7	2.5	1.3	0.5	2.8	0.4
HLA-C2	C*05:01	100.0	89.9	86.2	78.9	0.1	0.6	1.3	7.8	2.5	0.7	5.6	0.8

FIGURE 1 | KIR2DL-Fc fusion proteins display differential binding affinities to HLA-C expressing 721.221 cell lines. **(A)** Overview of the assessment of binding affinities between HLA-C expressing 721.221 cell lines and KIR2DL-Fc fusion proteins using flow cytometry. Created with BioRender.com. **(B)** Upper panel: Representative histograms depicting binding of KIR2DL1*001-Fc (orange), KIR2DL3*001-Fc (purple) and the respective secondary antibody (Ab) control (black, dotted) to 721.221 (.221),.221-C*07:01 (C1) and .221-C*02:02 (C2) cell lines. Expression was quantified as fluorescence intensity (x-axis). Lower panel: Cumulative data showing binding of KIR2DL-Fc fusion proteins to .221, .221-C*07:01 (C1) and .221-C*02:02 (C2) cell lines. Binding of KIR2DL1-Fc (orange), KIR2DL2-Fc (light purple) and KIR2DL3-Fc (dark purple) allotypes is displayed as relative fluorescence intensity (RFI) normalized to the secondary antibody only control and adjusted for HLA-ABC expression of the respective 721.221 cell lines. Box plots show median and 25%/75% percentile. Data points represent at least five technical replicates ($n \leq 6$). **(C)** Summarizing results of binding assays between 12 KIR2DL-Fc allotypes and .221s expressing 12 different HLA-C allotypes. Highest binding value was set to 100% and all other values were calculated in relation to the 100% value.

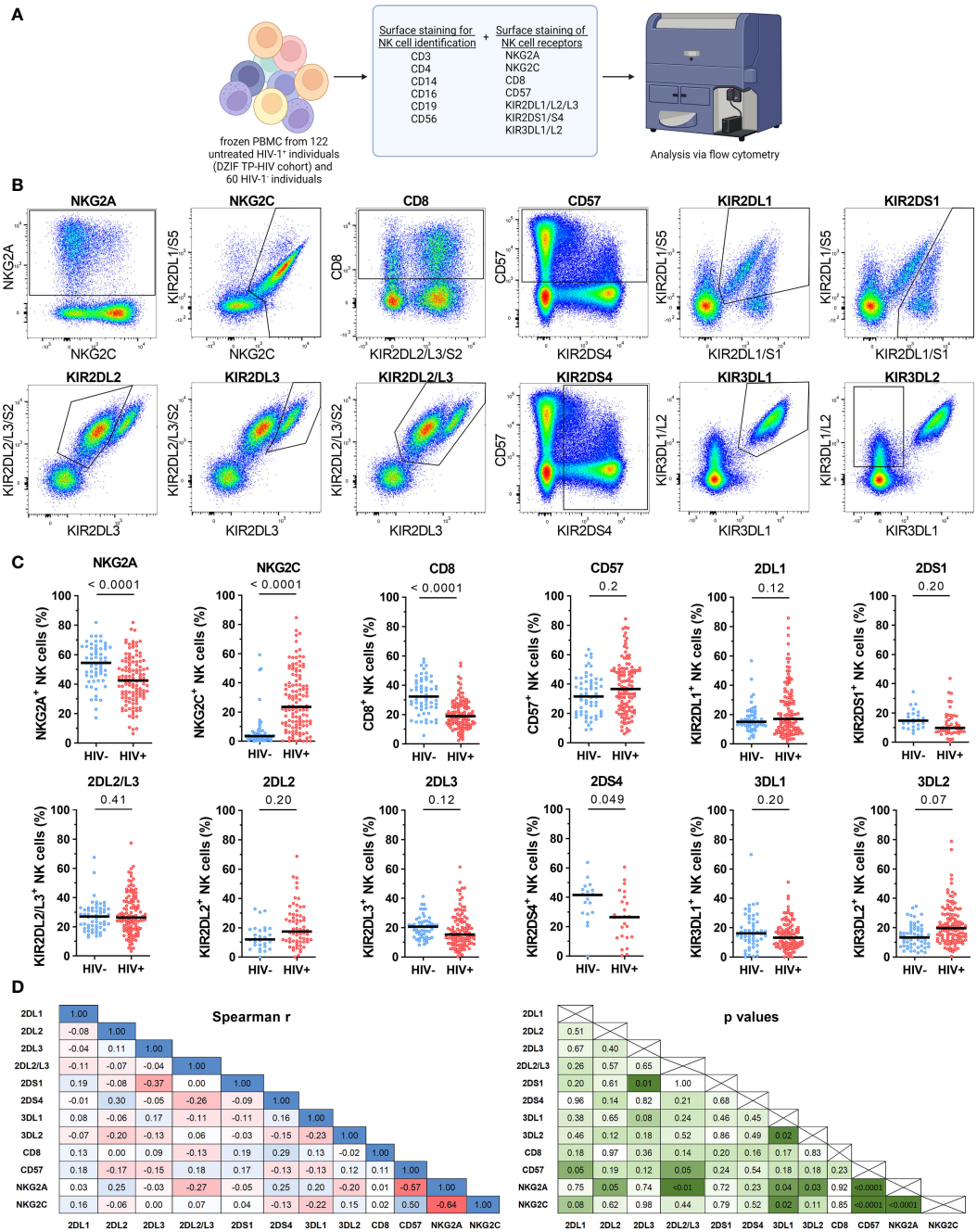


FIGURE 2 | Cell surface expression of NK cell receptors of HIV-1$^-$ and untreated HIV-1$^+$ individuals. **(A)** Overview of the flow cytometric assessment of NK cell receptor expression on NK cells from HIV-1$^-$ (n = 60) and untreated HIV-1$^+$ (n = 122) individuals. Created with BioRender.com **(B)** Respective expression patterns of the NK cell receptors NKG2A, NKG2C, CD8, CD57, KIR2DL1, KIR2DS1, KIR2DL2, KIR2DL3, KIR2DL2/L3, KIR2DS4, KIR3DL1 and KIR3DL2 on bulk NK cells as well as the respective gating. **(C)** Scatter plots displaying the percentage of NKG2A$^+$, NKG2C$^+$, CD8$^+$, CD57$^+$, KIR2DL1$^+$, KIR2DS1$^+$, KIR2DL2/L3$^+$, KIR2DL2$^+$, KIR2DL3$^+$, KIR2DS4$^+$, KIR3DL1$^+$ and KIR3DL2$^+$ cells within bulk NK cells in HIV-1$^-$ (blue) and HIV-1$^+$ (red) individuals. Each data point represents one donor. Donors lacking the respective gene, containing a gene deletion (KIR2DS4-del) or a null allele (KIR3DL1*004) were excluded (NKG2A/NKG2C/CD8/CD57/KIR2DL2/L3: HIV-1$^-$: n = 60, HIV-1$^+$: n = 122; KIR2DL1: n = 59, n = 118; KIR2DS1: n = 26, n = 47; KIR2DL2: n = 28, n = 64; KIR2DL3: n = 55, n = 111; KIR2DS4: n = 19, n = 25; KIR3DL1: n = 55, n = 108). **(D)** Correlation analyses between the frequencies of all tested receptor$^+$ NK cell subsets from HIV-1$^+$ individuals. Left panel: r_s values; right panel: p values. **Data information: (C)** Bars indicate the median for each group. Multiple linear regression analysis was used to determine differences between HIV-1$^-$ and HIV-1$^+$ individuals. **(D)** Spearman rank analysis. **(C, D)** p values were adjusted for multiple comparisons (Benjamini/Krieger/Yekutieli).

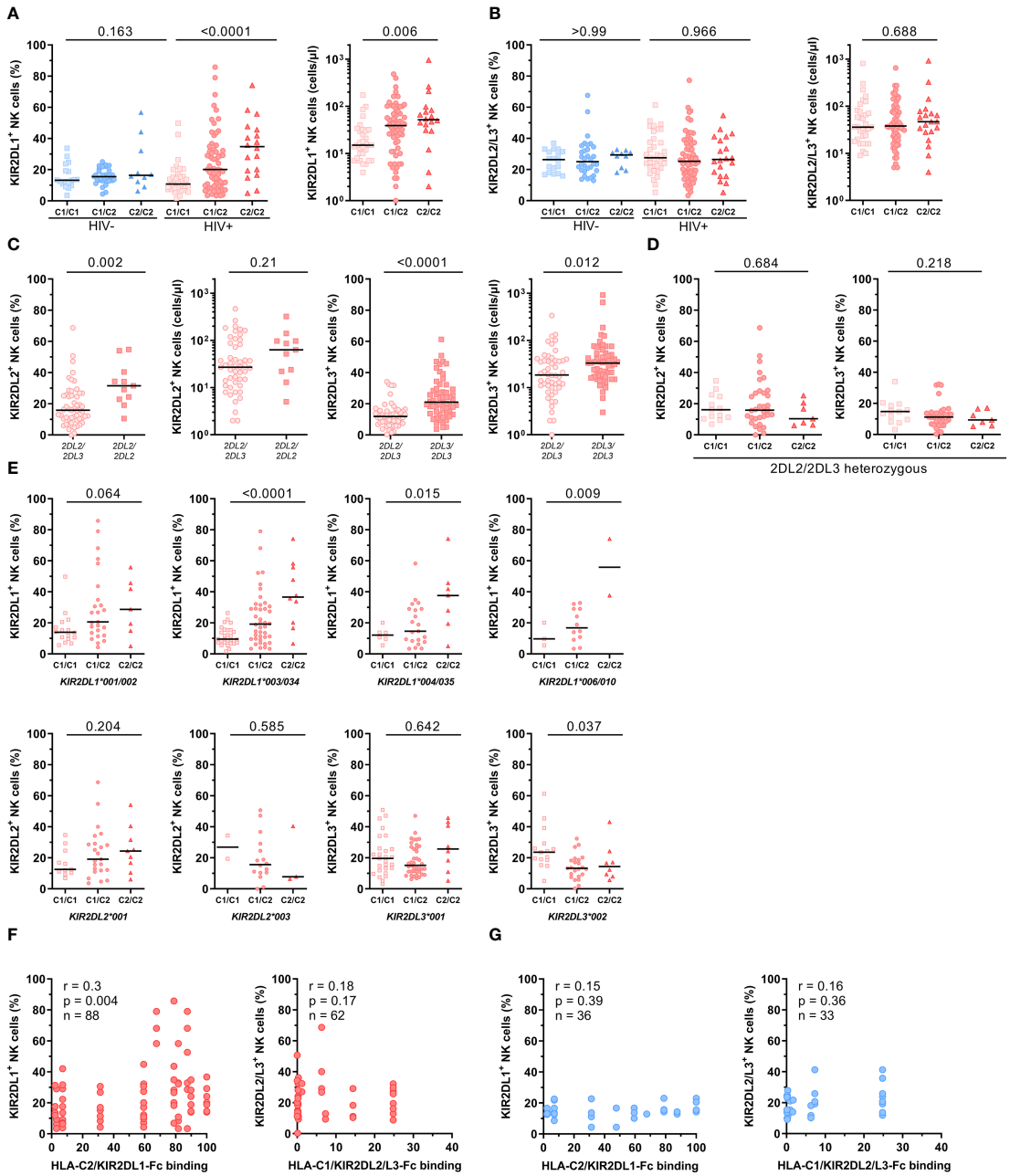


FIGURE 3 | Impact of *HLA-C* and *KIR* genotypes on the frequency of NK cells expressing HLA-C-binding KIRs. **(A)** Percentage of KIR2DL1$^+$ cells within bulk NK cells in HIV-1$^-$ and HIV-1$^+$ individuals (left panel). Absolute numbers of KIR2DL1$^+$ NK cells in HIV-1$^+$ individuals (right panel). **(B)** Percentage of KIR2DL2/L3$^+$ cells within bulk NK cells in HIV-1$^-$ and HIV-1$^+$ individuals (left panel). Absolute numbers of KIR2DL2/L3$^+$ NK cells in HIV-1$^+$ individuals (right panel). (A/B) Donors were stratified by *HLA-C* group genotypes. Genotypes were defined by the presence of HLA-C allotypes carrying either a C1 or C2 epitope (C1/C1 = C1^{homozygous}, C1/C1 = C1/C2^{heterozygous}, C2/C2 = C2^{homozygous}). **(C)** Percentage and absolute cell numbers of KIR2DL2$^+$ NK cells and KIR2DL3$^+$ NK cells in HIV-1$^+$ individuals stratified by the presence of *KIR2DL2* or *KIR2DL3* alleles. **(D)** Percentage of KIR2DL2$^+$ and KIR2DL3$^+$ NK cells in *KIR2DL2/L3*^{heterozygous} HIV-1$^+$ individuals stratified by *HLA-C* group genotypes. **(E)** Percentage of KIR2DL1$^+$, KIR2DL2$^+$ and KIR2DL3$^+$ NK cells in HIV-1$^+$ individuals stratified by *HLA-C* group genotypes and *KIR2DL* alleles. **(F)** Correlation between percentage of KIR2DL1$^+$ NK cells and HLA-C2/KIR2DL1-Fc binding, KIR2DL2/L3$^+$ NK cells and HLA-C1/KIR2DL2/L3-Fc binding in HIV-1$^+$ individuals (red) stratified by KIR/HLA binding affinities determined in **Figure 1**. **(G)** Correlation between percentage of KIR2DL1$^+$ NK cells and HLA-C2/KIR2DL1-Fc binding, KIR2DL2/L3$^+$ NK cells and HLA-C1/KIR2DL2/L3-Fc binding in HIV-1$^-$ individuals (blue) stratified by KIR/HLA binding affinities determined in **Figure 1. Data information:** Black bars display the median. **(A, B, D, E)** One-way ANOVA, test for linear trend. **(C)** Mann-Whitney test. P values were adjusted for multiple comparisons (**A, B, D:** Bonferroni; **E**: Benjamini/Krieger/Yekutieli). **(F, G)** Spearman rank correlation.

TABLE 3 | Demographic and clinical profile of HIV-1$^-$ and HIV-1$^+$ individuals.

	Number total	HIV-1$^-$ individuals 60	HIV-1$^+$ individuals 122
Demographic data	**Age in years**	N = 43	N = 122
	Median (Min; Max)	30 (22; 64)	36.5 (20; 73)
	Sex	N = 44	N = 122
	Male, number (%)	21 (47.7)	114 (93.4)
	Female, number (%)	23 (52.3)	8 (6.6)
Clinical data	**Viral load in copies/ml**	n.d	N = 121
	Median		516,000
	(Min; Max)		(40; 170,000,000)
	CD4 T cells in cells/μL	n.d	n=118
	Median (Min; Max)		438 (22; 2,601)
	CD4 T cells in %	n.d	n=116
	Median (Min; Max)		24.6 (3; 54)
	CD8 T cells in cells/μL	n.d	N = 84
	Median (Min; Max)		936 (177; 6,636)
	CD8 T cells in %	n.d	N = 79
	Median (Min; Max)		50 (29; 85.9)
	CD4/CD8 ratio	n.d	N = 84
	Median (Min; Max)		0.49 (0.1; 1.86)
	NK acells in cells/μL	n.d	N = 118
	Median (Min; Max)		164 (14; 2,004)

a marker for terminal differentiation, was slightly higher in HIV-1$^+$ individuals (36.7%) compared to the HIV-1$^-$ control group (31.5%) but did not reach statistical significance after adjustment for age and sex ($p = 0.2$). Percentage of CD57$^+$ NK cells was positively associated with the percentage of NKG2C$^+$ NK cells ($r_s = 0.5$, $p < 0.0001$) and negatively with the percentage of NKG2A$^+$ NK cells ($r_s = -0.57$, $p < 0.0001$; **Figure 2D**). The relative frequency of CD8$^+$ NK cells was reduced in HIV-1$^+$ individuals (18.9%) in comparison to HIV-1$^-$ donors (32.3%, $p < 0.0001$) but did not correlate with any other investigated NK cell receptor. For most KIRs expression patterns seemed to be unaffected by HIV-1 infection. No differences in the relative frequency of KIR2DL1$^+$ cells ($p = 0.12$) and KIR2DL2/L3$^+$ cells ($p = 0.41$) were observed, even after stratification into KIR2DL2 ($p = 0.2$) and KIR2DL3 ($p = 0.12$). The activating KIRs, KIR2DS1 ($p = 0.2$) remained unaltered as well but KIR2DS4 showed lower relative frequencies in HIV-1$^+$ individuals (26.5% *vs.* 41.9%, $p = 0.049$). The proportion of KIR3DL1$^+$ NK cells was not significantly altered ($p = 0.20$), even when adjusted for its ligand Bw4 or stratified by KIR3DL1 allotype groups (**Supplementary Figure 2**) in contrast to KIR3DL2, which exhibited increased relative frequencies in HIV-1$^+$ individuals (19.7% *vs.* 13.4%, $p = 0.07$). Analysis of relative frequencies and absolute cell numbers of the respective receptor$^+$ NK cells revealed a significant positive correlation for all receptors (**Supplementary Figure 3**), indicating that the observed changes represent an actual expansion or contraction of NK cell subsets.

Subsequently, we investigated whether the altered NK cell receptor repertoire was attributed to changes of the distribution of the three major CD56 NK cell subsets. Based on the expression levels of CD56, NK cells can be distinguished into CD56^{Bright} (CD16$^-$/dim), CD56^{Dim} (CD16$^+$) and CD56^{Negative} sub-populations (CD16$^+$), each displaying specific receptor profiles on their own (50). Compared to the HIV-1- control group, the frequency of CD56^{Bright} NK cells was significantly decreased within the NK cell compartment of HIV-1- infected individuals ($p < 0.0001$) (**Supplementary Figure 4**). Conversely, the proportion of CD56^{Negative} NK cells was increased ($p < 0.0001$). Subsequently, the three CD56 NK cell subsets were analyzed for the expression of the eleven NK cell receptors (**Table 4**). Changes of NK cell receptor profiles were observed in all CD56 NK cell subsets. In the CD56^{Bright} NK cell subset, eight of the investigated NK cell receptors, namely KIR2DL1, KIR2DL2, KIR2DL3, KIR2DS4, KIR2DS1, KIR3DL1, CD57 and NKG2C exhibited an increased frequency in HIV-1$^+$ individuals, although it should be noted that percentage point differences were rather marginal for all tested KIRS. In contrast, NKG2A and CD8 were significantly decreased. In the CD56^{Dim} subset, CD8$^+$ NK cells showed decreased frequencies, while the KIR3DL2$^+$ NK cell subset was enlarged. CD56^{Negative} NK cells showed similar changes in the expression patterns as the CD56^{Dim} subset in both HIV-1$^-$ and HIV-1$^+$ groups, although CD56^{Negative} NK cells displayed an overall lower percentage of receptor$^+$ cells compared to CD56^{Dim} NK cells. Taken together, we observed reduced percentages of CD8$^+$ NK cells in all CD56 NK cell subsets of HIV-1$^+$ individuals compared to HIV-1$^-$ individuals, whereas the NKG2C$^+$ subset was enlarged. In contrast, changes in the KIR profiles between HIV-1$^+$ and HIV-1$^-$ individuals were rather marginal in the CD56^{Dim} subset in which KIRs are predominantly expressed, with the exception of KIR3DL2.

HLA-C and *KIR2DL* Genotypes Impact Frequency of KIR2DL$^+$ NK Cells

To investigate the influence of *KIR2DL/HLA-C* genotype combinations on KIR2DL expression on NK cells, HIV-1$^+$ and HIV-1$^-$ individuals were stratified according to their *HLA-C* allotypes. HIV-1$^+$ individuals showed significant differences in the frequency of KIR2DL1$^+$ NK cells between the generated

TABLE 4 | NK cell receptor expression in NK cell subsets.

			$CD56^{Bright}$			$CD56^{Dim}$			$CD56^{Negative}$		
receptor	HIV- n	HIV+ n	HIV- median(25%/ 75% percentile)	HIV+ median(25%/ 75% percentile)	p value*	HIV- median(25%/ 75% percentile)	HIV+ median(25%/ 75% percentile)	p value*	HIV- median(25%/ 75% percentile)	HIV+ median(25%/ 75% percentile)	p value*
NKG2A	60	122	95.9 (94.1/97.1)	93.7 (89.7/96.9)	0.0003	52.5 (41.3/63.2)	41.4 (29.6/54.7)	0.002	32.9 (23.4/45.3)	29.9 (20.6/42.7)	0.1
NKG2C	60	122	10.2 (7.1/15.9)	14.0 (8.0/20.1)	0.04	2.6 (1.7/5.4)	24.9 (11.0/42.0)	<0.0001	1.8 (1.0/3.4)	17.1 (6.9/33.9)	<0.0001
CD8	60	122	23.8 (18.7/31.1)	17.2 (11.0/23.3)	0.003	37.1 (29.4/45.8)	19.5 (13.3/28.0)	<0.0001	28.6 (20.7/33.1)	14.3 (8.4/19.2)	<0.0001
CD57	60	122	0.5 (0.2/1.0)	0.8 (0.4/2.2)	0.0001	34.2 (23.4/46.2)	40.2 (26.0/52.9)	0.13	9.5 (6.4/13.8)	14.0 (7.5/27.1)	0.052
KIR2DL1	59	118	1.4 (0.8/1.9)	2.2 (1.2/4.0)	0.0003	17.9 (14.2/23.6)	18.9 (9.9/36.8)	0.36	10.4 (7.7/13.1)	9.3 (4.4/17.7)	0.45
KIR2DS1	26	47	0.9 (0.6/1.4)	1.5 (0.8/2.4)	0.006	16.5 (11.2/20.5)	9.8 (6.8/18.5)	0.052	11.6 (7.7/17.5)	8.2 (4.7/12.6)	0.056
KIR2DL2/ L3	60	122	1.9 (1.5/2.9)	3.2 (1.8/6.1)	0.0001	29.7 (21.2/33.8)	27.2 (20.0/39.0)	0.49	18.6 (12.6/24.0)	19.6 (12.3/28.1)	0.13
KIR2DL2	28	64	1.1 (0.6/1.9)	2.0 (1.1/3.4)	0.035	12.3 (8.3/19.6)	17.2 (10.8/29.8)	0.77	10.8 (5.8/15.8)	13.2 (7.6/22.3)	0.45
KIR2DL3	55	111	1.4 (0.8/1.9)	2.0 (1.1/3.5)	0.04	21.7 (14.4/25.5)	16.4 (10.1/26.3)	0.074	12.6 (8.8/17.6)	10.1 (6.1/18.9)	0.40
KIR2DS4	19	25	3.4 (2.1/4.3)	4.2 (1.9/8.8)	0.042	45.4 (30.5/49.7)	27.3 (10.3/40.3)	0.27	30.4 (20.1/37.9)	23.1 (8.2/29.8)	0.71
KIR3DL1	55	108	1.2 (0.6/2.0)	2.8 (1.5/4.3)	0.003	15.5 (9.0/24.0)	12.8 (8.0/20.4)	0.23	8.0 (4.7/12.6)	8.6 (5.1/14.1)	0.42
KIR3DL2	60	122	11.3 (7.3/15.0)	10.5 (6.9/13.8)	0.21	14.9 (9.0/22.9)	20.4 (13.2/29.2)	0.048	10.4 (6.4/16.6)	17.6 (10.5/27.1)	0.0003

*Multiple linear regression analysis (HIV, sex, age). p values were adjusted for multiple comparison (Benjamini/Krieger/Yekutieli).

groups, while no differences were detected in the HIV-1^- control group (**Figure 3A**). HIV-1^+ individuals carrying the cognate HLA-C2 ligand (C1/C2, C2/C2) showed an increased frequency of KIR2DL1^+ NK cells, whereas individuals without *HLA-C2* alleles (C1/C1) exhibited a generally lower percentage of KIR2DL1^+ NK cells (left panel, linear trend: $p < 0.0001$). This effect was not due to a skewed distribution of KIR2DL1-C^{245} and KIR2DL1-R^{245} allotypes between HIV-1^+ and HIV-1^- individuals or between *HLA-C2*$^+$ or *HLA-C2*$^-$ donors (data not shown), which was previously shown to affect KIR2DL1 expression (51). The observed gene-dose dependent effect of *HLA-C2* alleles within the HIV-1^+ group was also observed when using absolute cell numbers as a readout (right panel, $p = 0.006$). In contrast, presence of the HLA-C1 ligand did not affect the proportion or cell number of NK cells expressing the respective receptors KIR2DL2/L3 in either HIV-1^+ or HIV-1^- individuals (**Figure 3B**, percentage HIV-1^-: $p > 0.99$, HIV-1^+: $p = 0.97$, cell number: $p = 0.69$).

Given that potential changes of KIR2DL2/L3 frequencies between HIV-1^+ and HIV-1^- groups were masked by the previously observed opposing directions of KIR2DL2$^+$ and KIR2DL3$^+$ NK cell frequencies, we stratified KIR2DL2/3 frequencies based on their *KIR2DL2/L3* genotype (**Figure 3C**). NK cells from homozygous *KIR2DL2* individuals (KIR*2DL2/L2*) showed a significantly increased frequency of KIR2DL2$^+$ NK cells compared to heterozygous (*KIR2DL2/L3*) individuals independent of the underlying *HLA-C* genotype ($p = 0.002$). This effect was also present in KIR2DL3$^+$ NK cells when comparing *KIR2DL3* homozygous (*KIR2DL3/L3*) and heterozygous (*KIR2DL2/L3*) individuals ($p < 0.0001$). To exclude the skewing effect of *KIR2DL2* and *KIR2DL3* homozygosity, stratification of *HLA-C* allotypes was performed only for *KIR2DL2/L3* heterozygous HIV-1^+ individuals (**Figure 3D**). Increased frequencies of KIR2DL2$^+$ and KIR2DL3$^+$ NK cells in donors with two *HLA-C1* alleles (C1/C1) were observed compared to individuals lacking *HLA-C1* alleles (C2/C2), however the overall trend did not reach statistical significance (KIR2DL2$^+$: $p = 0.68$, KIR2DL3$^+$: $p = 0.22$).

Based on the observed effects of homo- or heterozygosity of the *KIR2DL/L3* gene, we sought to further stratify donors based on the specific *KIR2DL* alleles (**Figure 3E**). In line with the results for KIR2DL1^+ NK cells from all HIV-1^+ individuals stratified by their *HLA-C* allotypes, HIV-1^+ individuals with *KIR2DL1*001/002*, **003/*034*, **004/*035* or **006/010* alleles showed the same distribution of KIR2DL1^+ NK cells with the highest frequency of KIR2DL1^+ NK cells in *HLA-C2* homozygous individuals and the lowest frequency in *HLA-C1* homozygous donors. Moreover, individuals positive for *KIR2DL2*001* and with at least one *HLA-C2* allele (C1/C2, C2/C2) showed an increased frequency of NK cells expressing KIR2DL2 compared to individuals without *HLA-C2*. In contrast, individuals carrying a *KIR2DL2*003* allele showed reverse KIR2DL2 frequencies with the highest percentages for *HLA-C1/C1* homozygous and the lowest percentages for *HLA-C2/C2* homozygous individuals. Although, it should be noted that the sample size for this particular allele was rather low.

Stratification of *HLA-C* allotypes for different *KIR2DL3* alleles showed a slightly increased frequency of KIR2DL3$^+$ NK cells in *HLA-C2/C2* individuals positive for *KIR2DL3*001*, whereas individuals carrying a *KIR2DL3*002* allele had an increased frequency of KIR2DL3 in the group of *HLA-C1/C1* genotypes. In addition, the observed distribution of *KIR2DL1* alleles in HLA-C genotypes was also present but less pronounced when using absolute cell numbers, whereas *KIR2DL2* and *KIR2DL3* alleles showed no differences in *HLA-C* genotypes when using absolute cell numbers a s readout (**Supplementary Figure 5**). To examine a possible relationship between *KIR/HLA-C* genotype combinations and the frequency of KIR2DL$^+$ NK cells, correlation analyses between the frequencies of KIR2DL$^+$ NK cells and HLA-C/KIR2DL-Fc binding affinities were performed for HIV-1$^-$ and HIV-1$^+$ individuals (**Figures 3F, G**). For this, we included individuals heterozygous for *C1/C2* and carrying *HLA-C/KIR* combinations with corresponding binding data. Frequency of KIR2DL1$^+$ NK cells showed a significant positive correlation with the assessed binding affinities for HLA-C2/KIR2DL1 combinations (r_s = 0.3, p = 0.004) in HIV-1$^+$ individuals. On the other hand, KIR2DL1$^+$ NK cell frequency and HLA-C2/KIR2DL1 binding affinities for HIV-1$^-$ individuals, as well as KIR2DL2/L3$^+$ NK cell frequency and HLA-C1/KIR2DL2/L3 binding affinities for HIV-1$^+$ and HIV-1$^-$ individuals showed no significant correlation. Overall, these results suggest that both, the underlying HLA-C and KIR genotype of an individual has a direct impact on the expression levels of the corresponding KIR in the respective NK cell pool.

Host Genetics Impact *Vpu* Sequence Polymorphisms in Plasma and PBMC Samples

Lab-adapted HIV-1 strains and primary isolates display differential abilities to modulate HLA-C expression on the surface of infected CD4$^+$ T cells mediated by the accessory HIV-1 protein Vpu (29, 31). Previously, five amino acid (AA) positions have been identified at which specific residues were independently associated with HLA-C downregulation (30): Proline (P) at position 3, glutamic acid (E) at position 5, glycine (G) or threonine (T) at position 16 and serine (S) at position 24. Conversely, alanine (A) at position 15 negatively affected the ability to downregulate HLA-C. To investigate the impact of HLA-C/KIR2DL allotype combinations on Vpu sequence variations, genomic DNA and viral RNA from matching PBMC and plasma samples from 122 HIV-1$^+$ individuals were isolated and sequenced with next-generation sequencing (NGS) for Vpu (**Figure 4A**). Sequences of *Vpu* from 93 plasma and 67 PBMC samples passed quality control, were translated to amino acid sequences and then aligned to the Vpu protein from the lab-adapted HIV-1 strains NL4-3 and JR-CSF (**Figure 4B**). Comparison of sequence similarity for the five analyzed AA positions between matching PBMC and plasma samples showed a frequency of matching AA residues between 0.64 and 0.92, with the lowest frequency for position 16 and the highest frequency for position 5. In addition, the frequency of all observed residues for the AA position was determined for PBMC and plasma samples. For position 3, the most common AA was proline (PBMC: 0.69, plasma: 0.61) followed by serine and leucine. Position 5 showed the highest variations in AA residues with the highest frequency for isoleucine (PBMC: 0.63, plasma: 0.45). The most common AA at position 15 was alanine (PBMC: 0.92, plasma: 0.73) followed by valine. Moreover, position 16 had comparable frequencies for alanine (PBMC: 0.29, plasma: 0.36), isoleucine (PBMC: 0.28, plasma: 0.14) and glycine (PBMC: 0.33, plasma: 0.36). Position 24 showed the lowest variations in AA residues with a serine as the most common AA (PBMC: 0.61, plasma: 0.63) followed by a threonine.

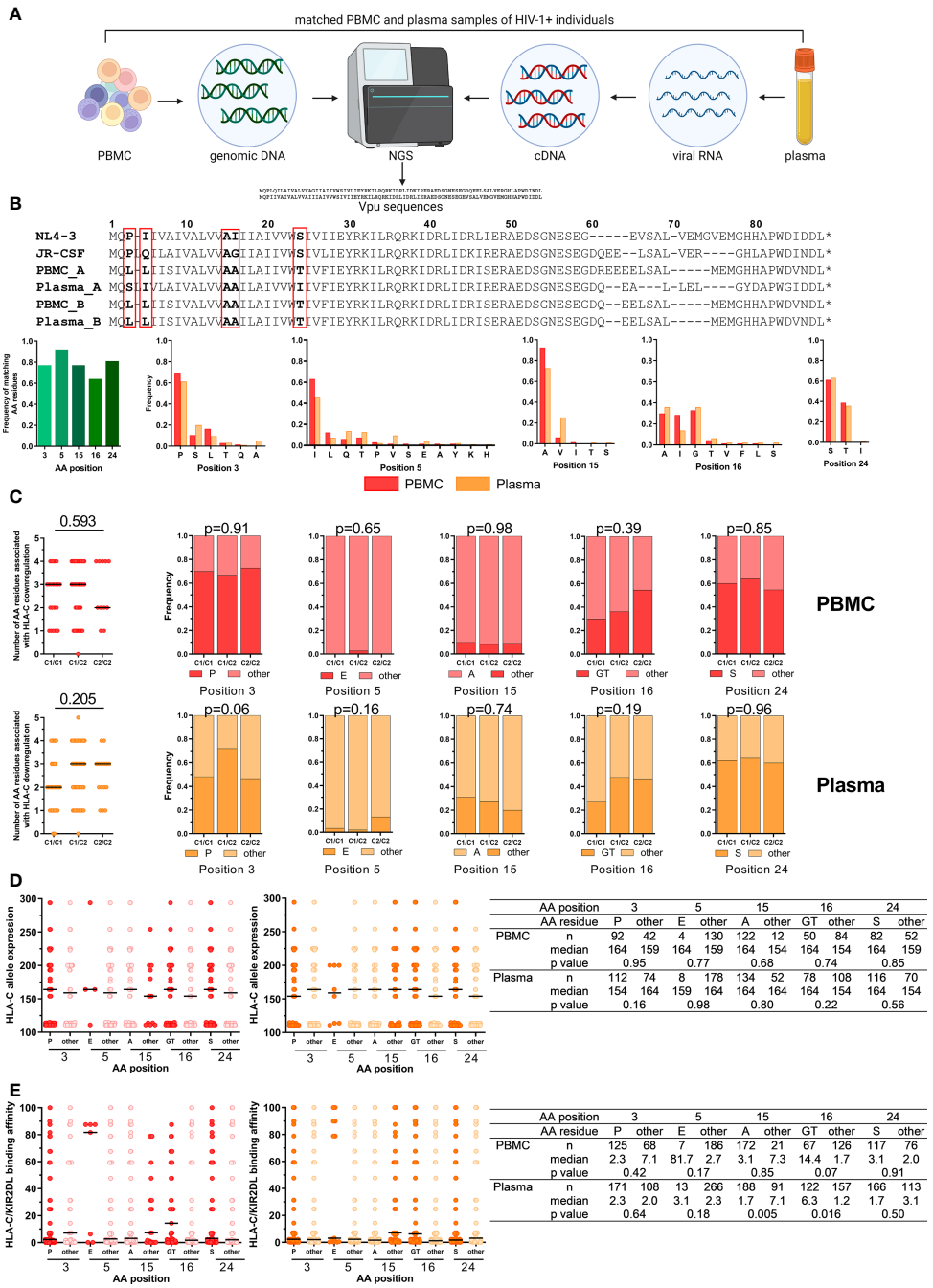
Stratification of HLA-C allotypes was performed for Vpu sequences for PBMC and plasma samples and analyzed for the five respective AA previously identified to influence HLA-C expression levels (**Figure 4C**). Vpu sequences isolated from PBMC samples from HLA-C1$^+$ individuals contained a median of three of the five AA residues, whereas *HLA-C2* homozygous individuals had a median of two AA residues that were associated with HLA-C downregulation (linear trend, p = 0.593). In comparison, Vpu sequenced from viral RNA from plasma samples had three of the five AA residues in individuals with at least one *HLA-C2* allele and *HLA-C1* homozygous individuals had two (p = 0.205). However, there were no differences detectable in the frequency of AA residues associated with HLA-C downregulation when individuals were stratified by their *HLA-C* genotype.

HLA-C surface expression levels vary and correlate with the HLA-C allotype (28). To identify a potential influence of HLA-C surface expression levels on Vpu sequence variations, Vpu sequences from PBMC and plasma samples were analyzed for the five AA positions that are involved in HLA-C downregulation based on the average MFI HLA-C allele expression (**Figure 4D**). Our analysis showed no significant influence of *HLA-C* allele expression on individual AA residuces.

Finally, differences in AA residues for the five positions were analyzed based on the assessed binding affinities of HLA-C1/KIR2DL2/3 and HLA-C2/KIR2DL1 allotype combinations (**Figure 4E**). HLA-C/KIR2DL combinations with stronger binding affinities were more common in Vpu sequences from plasma samples with AA other than an alanine at position 15 (15other) (p = 0.005) or a glycine or threonine at position 16 (16GT) (p = 0.016) whereas HLA-C/KIR2DL binding affinities were not associated with specific amino acid residues in Vpu sequences from PBMC samples. Overall, these results indicated that HLA-C expression level and HLA-C/KIR2DL binding may have an influence on Vpu sequence polymorphisms.

DISCUSSION

In recent years multiple studies have drawn attention to the potential role of HLA-C and its cognate inhibitory receptors, KIR2DL1, KIR2DL2, and KIR2DL3, in the intrinsic control of HIV-1 infection. Most notably were two studies showing the protective effect of high HLA-C expression levels on HIV-1



	AA position	3		5		15		16		24	
	AA residue	P	other	E	other	A	other	GT	other	S	other
PBMC	n	92	42	4	130	122	12	50	84	82	52
	median	164	159	164	159	164	154	164	154	164	159
	p value	0.95		0.77		0.68		0.74		0.85	
Plasma	n	112	74	8	178	134	52	78	108	116	70
	median	154	164	159	164	164	164	164	154	164	154
	p value	0.16		0.98		0.80		0.22		0.56	

	AA position	3		5		15		16		24	
	AA residue	P	other	E	other	A	other	GT	other	S	other
PBMC	n	125	68	7	186	172	21	67	126	117	76
	median	2.3	7.1	81.7	2.7	3.1	7.3	14.4	1.7	3.1	2.0
	p value	0.42		0.17		0.85		0.07		0.91	
Plasma	n	171	108	13	266	188	91	122	157	166	113
	median	2.3	2.0	3.1	2.3	1.7	7.1	6.3	1.2	1.7	3.1
	p value	0.64		0.18		0.005		0.016		0.50	

FIGURE 4 | Impact of *HLA-C/KIR2DL* genotypes on Vpu sequence polymorphisms. **(A)** Overview of the work flow for Vpu sequencing from matched PBMC (n = 122) and plasma samples (n = 122) of untreated HIV-1$^+$ individuals. Created with BioRender.com **(B)** Upper panel: Representative alignment of amino acid (AA) sequences of HIV-1 Vpu passing quality control. Lower panels: Frequency of matching AA residues at position 3, 5, 15, 16 and 24 between matched PBMC and plasma samples (n = 53). Frequency of individual AA residues at 3, 5, 15, 16 and 24 in PBMC (n = 67) and plasma (n = 93) samples. **(C)** Left: Dot plots displaying the cumulative number of AA residues associated with Vpu-mediated HLA-C downregulation per sequence. Right: Frequency of AA residues at position 3, 5, 15, 16 and 24. Individuals were stratified by *HLA-C* group genotypes (x-axis). Upper panel: PBMC, lower panel: Plasma. **(D)** Expression levels of HLA-C alleles of HIV-1$^+$ donors. Data points are displayed for each position (3, 5, 15, 16 and 24) and AA residues. AA residues associated with HLA-C downregulation are displayed in red (PBMC, left panel) or dark orange (plasma, right panel). Summary table displaying descriptive and comparative statistics. **(E)** Binding affinities of KIR/HLA-C combinations of HIV-1$^+$ donors. Data points are displayed in red (PBMC, left panel) or dark orange (plasma, right panel). Summary table displaying descriptive and comparative statistics. **Data information:** Black bars display the median. **(C)** Left panel: One-way ANOVA, test for linear trend. Right panels: Chi square test. **(D, E)** Mann-Whitney test.

progression (28) and the differential ability of HIV-1 to modulate HLA-C expression through the accessory protein Vpu (29). In contrast to Nef-mediated downregulation of HLA-A/B, the magnitude of HLA-C modulation by Vpu varies extensively across viral strains. Further investigations revealed that HIV-1 adapts its ability for downmodulation to the *HLA-C* genotype of the host (30). However, the majority of the investigated viral strains exhibited only a marginal ability to reduce HLA-C expression on infected cells (30) indicating that other factors than escape from CTL recognition through HLA-C downmodulation may contribute to Vpu sequence polymorphisms. Previous reports by our group demonstrated that KIR2DL$^+$ NK cells are able to sense HIV-1-mediated alterations of HLA-C (31). Based on these studies, we hypothesized that differential binding affinities across KIR and HLA-C allotypes in part pre-determine KIR2DL$^+$ NK cell induced immune pressure and Vpu-associated viral escape.

Our results demonstrated a hierarchy of affinities along the various combinations and confirmed the binding specificities of KIR2DL1 (HLA-C2), KIR2DL3 (HLA-C1) and KIR2DL2 (HLA-C1/-C2) (52). These results are largely consistent with those from the cell-free system of Hilton et al. who used KIR-Fc fusion constructs and microbeads, each coated with a different HLA class I allotype (39). While specificities between KIR2DL and HLA-C are defined by position 44 of the KIR protein (15) and position 80 of the α1 domain of HLA-C (7), other amino acid positions within both molecules fine-tune avidity (19, 53). Data on the functional consequences of the variegated affinities in the context of HIV-1 infection have been limited to KIR3DL1 (54, 55). However, multiple studies have shown that changes in the HLA class I presented peptidome can modulate binding to KIRs and subsequently impact NK cell functions (33, 56, 57). Moreover, HIV-1 sequence polymorphisms have been associated with *KIR2DL* genes alone and in combination with HLA-C indicating NK cell-mediated immune pressure involving KIR2DL$^+$ NK cells (36, 37). It is therefore conceivable that binding affinities predetermined by *KIR2DL/HLA-C* genotypes may have a similar impact.

In this context, expansion of KIR$^+$ NK cell subsets can be an indicator for the involvement of specific KIR$^+$ NK cell subsets in the antiviral immune response against HIV-1 (58–60). HIV-1 infection has been associated with significant changes in the NK cell receptor repertoire (61–63). In our phenotypical characterization we investigated the expression of NK cell receptors interacting with various HLA class I molecules in a cohort of untreated, viremic HIV-1$^+$ individuals. Noteworthy, decreased numbers of CD8$^+$ NK cells in HIV-1-infected individuals have been observed in a previous study, where it correlated positively with HIV loads and inversely with CD4$^+$ T cell counts (64). We also observed an overall contraction of the NKG2A$^+$ NK cell subset which was closely tied to an expansion of NKG2C$^+$ and CD57$^+$ NK cells in the same donors. The inhibitory NKG2A receptor interacts with the non-classical HLA class I molecule HLA-E on target cells, preventing healthy cells from NK cell-mediated lysis. Additionally, HLA-E is recognized by NKG2C, which delivers an activating signal to the NK cell (65). NKG2A$^+$ NK cells have been implicated to possess superior anti-HIV activity compared to other subsets, whereas KIR2DL$^+$ NK cells had a diminished ability to degranulate in response to HIV-1-infected CD4$^+$ T cells even in the absence of NKG2A (66). In line with our results, Mavilio et al., showed a decreased expression of NKG2A in HIV-1-infected viremic individuals, which was associated with reduced inhibitory function in a redirected killing assay (61). Loss of NKG2A$^+$ NK cells corresponded with a high expansion of NK cells expressing NKG2C (67). In contrast, higher NKG2A expression levels in cytotoxic NK cell subsets have been reported in individuals with late stage HIV-1 infection (68). Expansion of NKG2C$^+$ and CD57$^+$ NK cells is not unique to HIV-1 infection (69). Similar changes have been observed for CMV and Hantavirus infection (70–72). It is still under investigation if these CD57$^+$NKG2C$^+$ NK cells have memory-like features specific for viral infections (73). Data on CMV seropositivity was not available for this cohort and therefore we cannot rule out a CMV-associated expansion in the HIV-1$^+$ cohort. However, an earlier study of our group indicated a CMV-independent, HIV-1-driven expansion of KIR2DL$^+$ NK cells (32).

Analysis of KIR$^+$ NK cells recognizing HLA-C initially showed a decrease of cells expressing the activating receptor KIR2DS4, while percentage of most inhibitory receptors remained unchanged. KIR2DS4 binds a subset of HLA-C1 and -C2 allotypes and HLA-A*11 (18) however its role in disease progression and NK cells regulation is not fully understood. KIR2DS4 has been associated with high viral loads and promotion of HIV-1 pathogenesis in chronic HIV-1 infection, probably through excessive NK cell activation (74, 75). HIV-1$^+$ individuals showed a higher frequency of the inhibitory KIR3DL2 receptor, which recognizes only HLA-A3 and -A11 in a peptide specific manner (11) and HLA-B27 (76). So far, not much is known about the role of KIR3DL2 in HIV-1 infection but other studies showed also an increased expression of KIR3DL2 in chronic HCV patients (77) and on activated NK cells from patients with spondylarthritis (78). Further stratification of our data based on *HLA-C* and *KIR2DL* genotypes indicated that the HIV-1-associated changes in the proportion of KIR2DL$^+$ NK cells within the NK cell pool were predetermined by host genetics. The frequency of KIR2DL1$^+$ NK cells was linked to the number of the cognate *HLA-C2* alleles, which we showed previously for individuals with primary HIV-1 infection (32). The increased frequency is potentially related to an increased surface expression of the respective HLA-C2 ligand. In addition, differentiation of *KIR2DL1* alleles displayed a similar "gene-dose" effect for the analyzed *KIR2DL1* alleles with the exception of *KIR2DL1*001/002*. Further analysis showed a positive correlation between KIR2DL/HLA-C binding affinities and the frequency of the respective KIR2DL$^+$ NK cell subset, thus further corroborating our hypothesis. However, we cannot rule out that the differences in the relative frequency of NK cells expressing various *KIR2DL* allotypes is due to changes in non-coding regions that may impact expression on an individual cell and within an individual NK cell pool (79–81).

Lastly, we investigated the impact of KIR/HLA-C genotypes on Vpu sequence polymorphisms. Molecular characterization of Vpu sequences of primary viruses identified five amino acid positions (3, 5, 15, 16 and 24) in the transmembrane and extracellular domain of Vpu that are associated with HLA-C downregulation (30). Grouping the amino acid position and residues based on their *HLA-C* genotype showed no differences in the frequency of amino acid positions/residues that downregulate HLA-C. We further tested whether differential *HLA-C* allele expression or HLA-C/KIR2DL binding were associated with AA residues that are involved in HLA-C downmodulation (28, 30). We observed an increased frequency of glycine or threonine at AA position 16 in individuals with stronger HLA-C/KIR2DL binding combinations compared to other amino acid residues at position 16, hinting that the binding affinities between KIR2DL and HLA-C molecules might be an additional factor impacting Vpu sequence polymorphisms. It should be noted that the observed signals contain a certain level of uncertainty that are attributed to the numerous allotype combinations in the cohort and the ambiguity of *KIR* allele typing. In general, the enormous diversity in HLA-C/KIR2DL binding combinations and different affinities, influencing the activation of NK cells and immune evasion mechanisms of HIV-1, make it challenging to develop a prediction model for specific Vpu sequence polymorphisms and consequences for NK cell activation based on *HLA-C/KIR2DL* genotypes.

Altogether, this study demonstrates the significant effects of HIV-1 infection on the NK cell pool in viremic, untreated HIV-1^+ individuals and provides evidence that the specific changes in the KIR2DL repertoire are predetermined by the underlying *KIR2DL/HLA-C* genotypes. The results also make a case that high resolution *KIR* typing and the generation of KIR/HLA binding models may be warranted to improve disease models and to potentially predict NK cell-associated immune responses and disease progression in various pathological settings and subsequently NK cell-based immune therapies.

DATA AVAILABILITY STATEMENT

Sequence data presented in the study are deposited in the European Nucleotide Archive (ENA), accession number #PRJEB53333. Storage of other raw data is performed by the Leibniz Institute of Virology on an internal server. Raw data will be made available upon request and can be shared after confirming that data will be used within the scope of the originally provided informed consent.

ETHICS STATEMENT

For this study, residual amounts of anonymized peripheral blood samples were used which were routinely taken from healthy blood donors at the the Institute for Transfusion Medicine, University Medical Center Hamburg-Eppendorf, Hamburg, Germany, and would have been discarded otherwise. All blood donors gave their general written consent to usage of their blood samples for scientific studies in an anonymized form. The anonymized use of human material complies with a vote by the ethics committee of the German Medical Association. Healthy blood donors recruited at the University Medical Center Hamburg-Eppendorf, Hamburg, Germany, provided written informed consent and studies were approved by the ethics committee of the Ärztekammer Hamburg (PV4780). Donors recruited through the Translational Platform HIV (TP-HIV) Cohort by the German Center for Infection Research (DZIF) provided written informed consent and the study was approved by the ethics committee of the Ärztekammer Hamburg (MC-316/14).

AUTHOR CONTRIBUTIONS

Conceptualization: CK; Funding acquisition: CK, MA, and AH; Methodology: CK, SVo, AN, and AH. Validation: SVo and AL; Formal Analysis: CK, SVo, SVi, and LR; Investigation: SVo, AL, TT, SB, DI, and JN; Resources: SVo, AN, PF, MA, GS, SP, GMNB, CL, AM, RP, NP, JR, SS, CS, CDS, EW, CW, PJN, JS, and AHS; Writing – original draft: CK and SVo; Writing - review and editing: all authors; Visualization: CK and SVo; Supervision: CK. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.922252/full#supplementary-material

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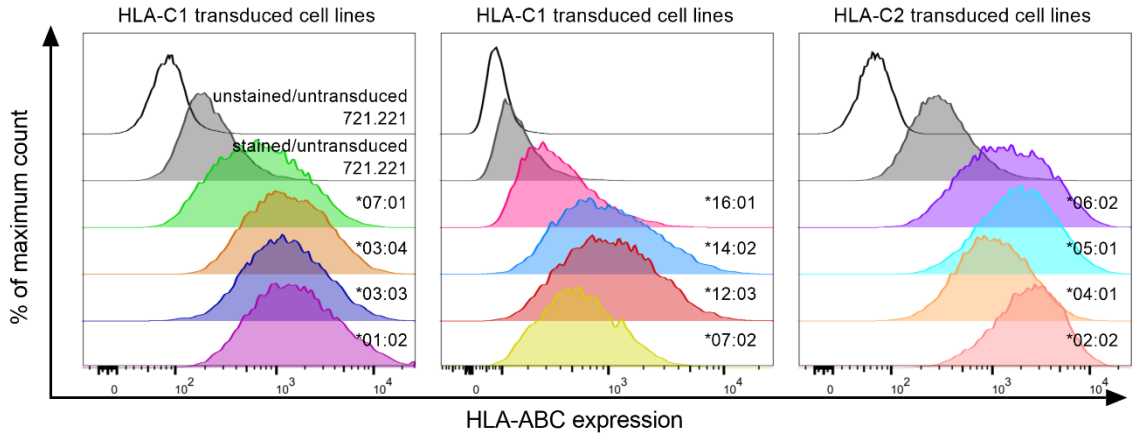
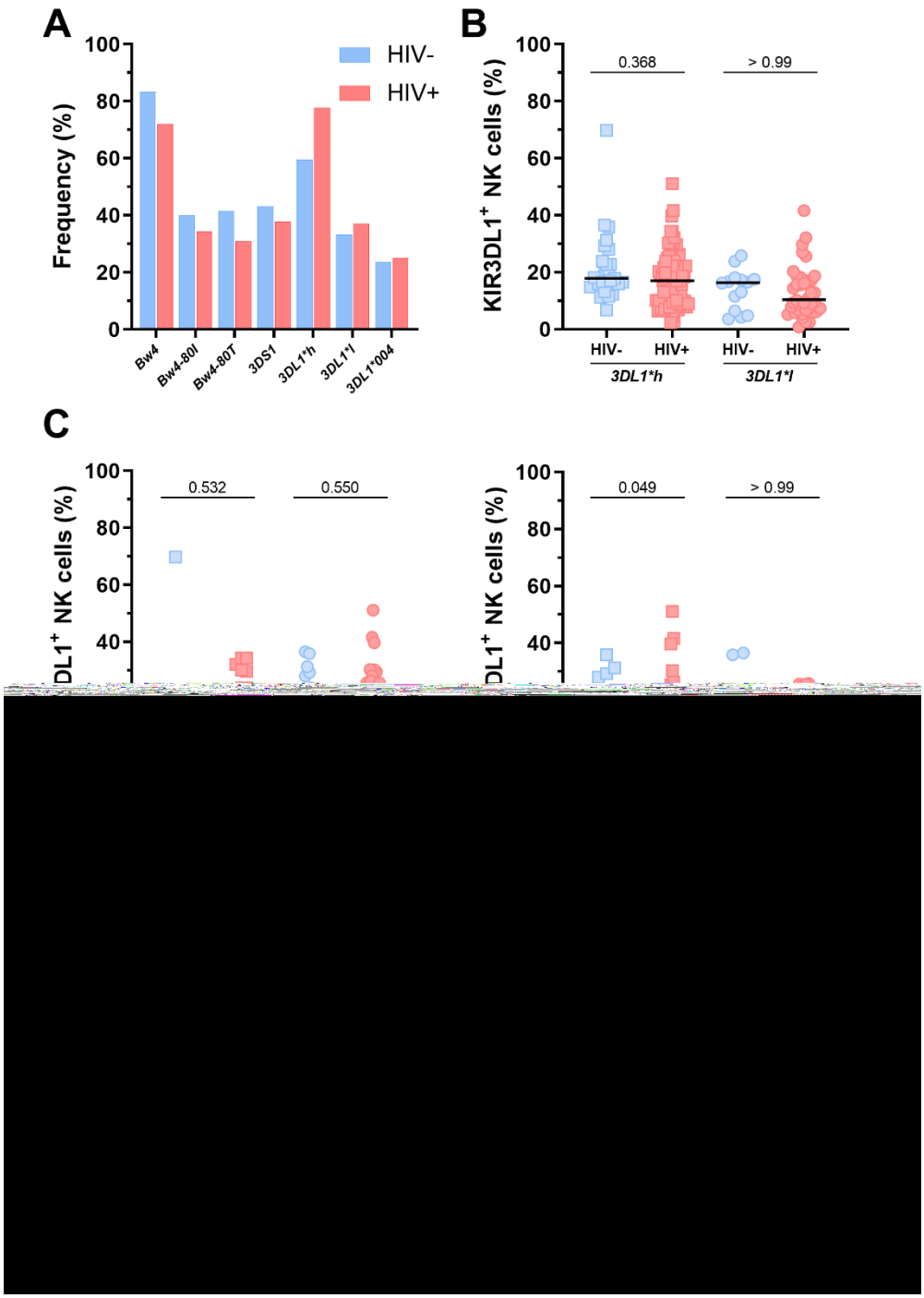
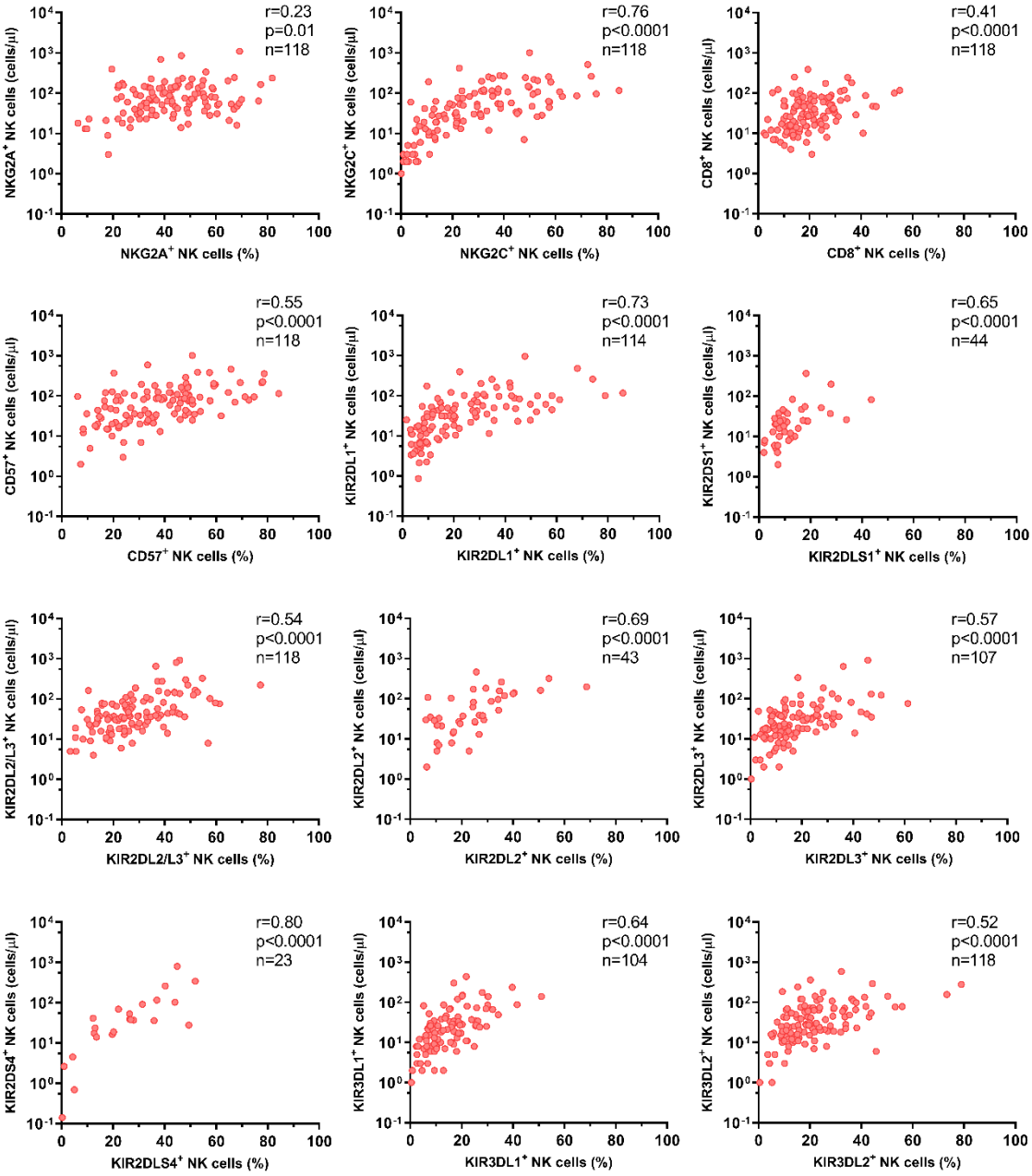
Supplementary Material



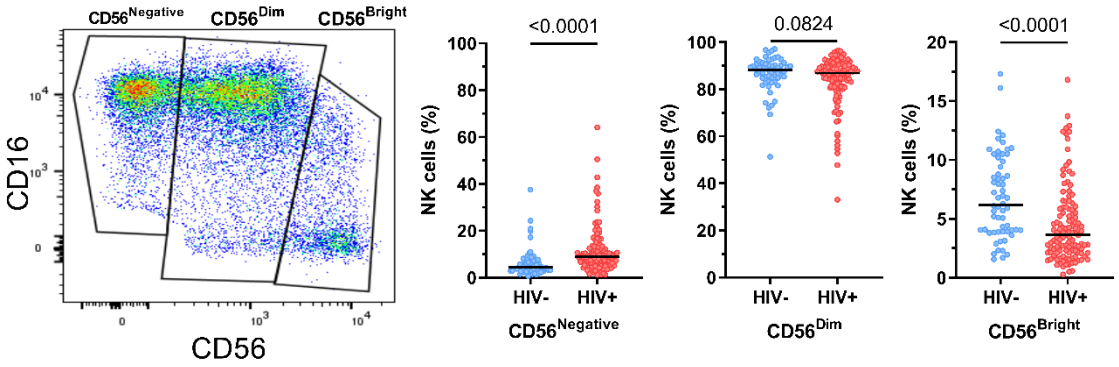
Supplementary Figure 1 | HLA-ABC expression of untransduced and HLA-C transduced 721.221 cell lines. All three histograms compare the HLA-ABC expression measured by flow cytometry (clone W6/32) of HLA-C transduced 721.221 cell lines to unstained (black, not filled) and stained (grey, filled) untransduced 721.221 cells. The left and middle histograms show the expression of the eight HLA-C1 transduced cell lines (HLA-C*01:02, *03:03, *03:04, *07:01, *07:02, *12:03, *14:02 and *16:01). The right histogram shows the HLA-C expression of the generated HLA-C2 cell lines (HLA-C*02:02; *04:01, *05:01 and *0602).



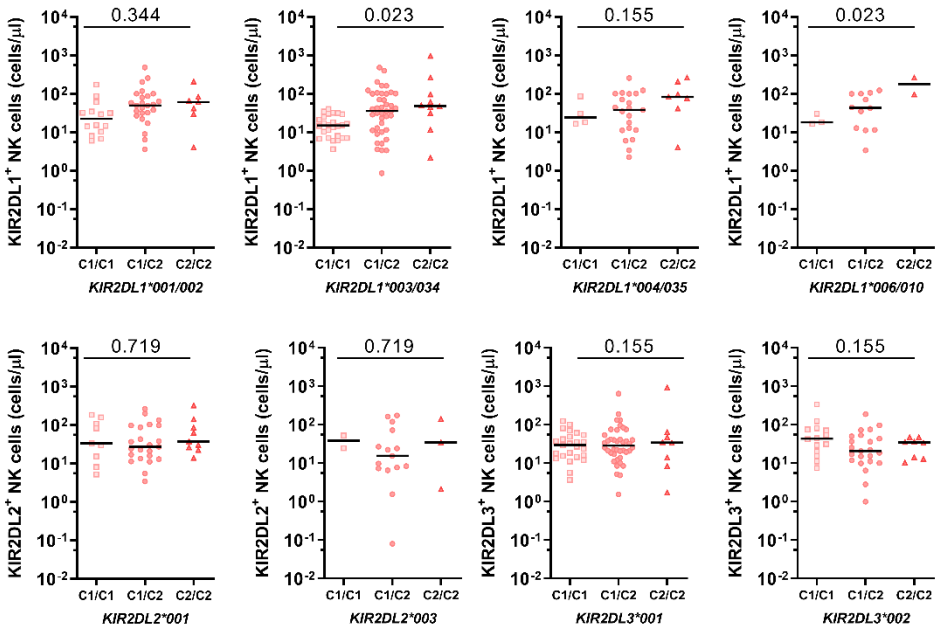
Supplementary Figure 2 | Stratification of KIR3DL1 allotype groups. (A) Frequency of *HLA-B Bw4* subtypes, *KIR3DS1*, *KIR3DL1*h* (high expressed alleles: *001, *002, *008, *009 and *015), *KIR3DL1*l* (low expressed alleles: *005, *007) and *KIR3DL1*004* alleles in HIV-1$^-$ (blue) and HIV-1$^+$ individuals (red). **(B)** Scatter plots display the percentage of KIR3DL1$^+$ bulk NK cells stratified by *KIR3DL1*h* and *KIR3DL1*l* alleles. **(C)** Scatter plots display the percentage of KIR3DL1$^+$ bulk NK cells of donors with *KIR3DL1*h* alleles stratified by Bw4 subtypes. **(D)** Scatter plots display the percentage of KIR3DL1$^+$ bulk NK cells of donors with *KIR3DL1*l* alleles stratified by Bw4 subtypes. (B-D) Mann-Whitney test. p values were adjusted for multiple comparisons (Bonferroni).



Supplementary Figure 3 | Cell numbers of NK cell subpopulations correlate positively with relative frequency of the respective receptor⁺ NK cells. Correlation between percentage of receptor⁺ NK cells and absolute cell numbers of NK cells expressing the receptors NKG2A, NKG2C, CD57, CD8, KIR2DS1, KIR2DL1, KIR2DL2/L3, KIR2DL2, KIR2DL3, KIR2DS4, KIR3DL1 and KIR3DL2 in HIV-1⁺ individuals. Spearman rank correlation analysis. p values were adjusted for multiple comparisons (Benjamini/Krieger/Yekutieli).



Supplementary Figure 4 | HIV-1 infection is associated with changes of the CD56 NK cell subset distribution. Exemplary flow cytometry plot showing gating of $CD56^{Negative}$, $CD56^{Dim}$, and $CD56^{Bright}$ NK cells. Dot plots displaying the relative frequency of $CD56^{Negative}$, $CD56^{Dim}$, and $CD56^{Bright}$ cells (left to right) within bulk NK cells from HIV-1^{-} (n = 60) and untreated HIV -1^{+} (n = 122) individuals. Bars indicate the median for each group. Mann-Whitney test was used to determine differences between HIV-1^{-} and HIV-1^{+} individuals.



Supplementary Figure 5 | Cell number of KIR2DL1$^+$, KIR2DL2$^+$ and KIR2DL3$^+$ NK cells in HIV-1$^+$ individuals. KIR2DL1$^+$, KIR2DL2$^+$ and KIR2DL3$^+$ NK cells of HIV-1$^+$ individuals were stratified by HLA-C group genotype and *KIR2DL* alleles. One-way ANOVA, test for linear trend was used to determine differences between *HLA-C group genotypes*. P values were adjusted for multiple comparisons (Benjamini/Krieger/Yekutieli).

Supplementary Table 1: Descriptive statistics for all eleven analyzed NK cell receptors.

	HIV-	HIV+	HIV-	HIV+	HIV-	HIV+		
receptor	**n**	**n**	**median (25%/75% percentile)**	**median (25%/75% percentile)**	**Min/Max**	**Min/Max**	**p value***	**adjusted p value***
NKG2A	60	122	54.4 (45.28/64.25)	42.50 (31.65/53.48)	17.2/81.8	6.44/81.9	<0.0001	<0.0001
NKG2C	60	122	3.47 (1.63/5.54)	23.45 (11.03/84.8)	0.1/56.3	0.1/84.8	<0.0001	<0.0001
CD8	60	122	32.3 (24.58/42.2)	18.9 (13.58/26.13)	5.74/57.9	2.34/55.1	<0.0001	<0.0001
CD57	60	122	31.5 (20.2/42.33)	36.7 (23.55/49.25)	8.85/63.6	6.14/84.4	0.17	0.20
KIR2DL1	59	118	15.0 (12.4/19.4)	17.0 (9.22/31.75)	3.55/56.7	1.6/85.8	0.07	0.12
KIR2DS1	26	47	14.75 (10.6/19.43)	9.86 (6.98/17.1)	6.18/34.6	0.04/43.6	0.23	0.20
KIR2DL2/L3	60	122	27.0 (19.35/32.23)	26.3 (18.7/37.35)	12.9/67.6	3.3/77.3	0.52	0.41
KIR2DL2	28	64	12.1 (7.49/18.73)	17.5 (10.63/28.5)	0.1/32.9	0.1/68.7	0.24	0.20
KIR2DL3	55	111	20.7 (13.7/23.9)	15.3 (9.6/24.4)	8.2/41.3	0.3/61.3	0.09	0.12
KIR2DS4	19	25	41.6 (29.30/45.30)	26.5 (12.85/38.6)	0.06/63.8	0.3/60.7	0.021	0.049
KIR3DL1	55	108	16.2 (11.1/23.9)	13.25 (7.8/19.68)	0.2/69.8	0.1/51.0	0.24	0.20
KIR3DL2	60	122	13.4 (7.83/19.2)	19.65 (12.48/28.75)	2.96/34.7	0.48/78.9	0.037	0.070

* Multiple linear regression analysis (HIV, sex, age), adjustment for multiplicity (Benjamini, Krieger and Yekutieli).