Impact of Interactions Between Killer Cell Immunoglobulin-like Receptors and their Non-Classical Cellular Ligands on the Antiviral Activity of NK Cells

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Submitted by

Pia Fittje

From Hannover, Germany

Hamburg

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Thesis director: Prof. Dr. Marcus Altfeld

Research Department Virus Immunology
Leibniz Institute of Virology
Hamburg, Germany

Dissertation reviewers:

First reviewer: **Prof. Dr. Marcus Altfeld**,
Research Department Virus Immunology, Leibniz Institute of Virology, Hamburg, Germany

Second reviewer: **Prof. Dr. Thomas Dobner**,
Research Department Viral Transformation, Leibniz Institute of Virology, Hamburg, Germany

Submitted by: **Pia Fittje**
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Höfle J, Trenkner T, Kleist N, Schwane V, Vollmers S, Barcelona B, Niehrs A, Fittje P, Huynh-Tran VH, Sauter J, Schmidt AH, Peine S, Hoelzemer A, Richert L, Altfeld M, Körner C. Engagement of TRAIL triggers degranulation and IFNγ production in human natural killer cells. *EMBO Rep*. 2022 Jun 27.

Koyro TF, Kraus E, Lunemann S, Hölzemer A, Wulf S, Jung J, Fittje P, Henseling F, Körner C, Huber TB, Grundhoff A, Wiech T, Panzer U, Fischer N, Altfeld M. Upregulation of HLA-F expression by BK polyomavirus infection induces immune recognition by KIR3DS1-positive natural killer cells. *Kidney Int*. 2021 May;99(5):1140-1148.

List of abbreviations

2ry	Secondary IgG antibody
ADCC	Antibody dependent cell-mediated cytotoxicity
AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cells
ART	Antiretroviral therapy
BKPyV	BK polyomavirus
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
CIITA	Class II major histocompatibility complex transactivator
DAA	Direct antiviral agents
DC	Dendritic cell
DDR	DNA damage responses
DNA	Deoxyribonucleic acid
DNAM-1	DNAX accessory molecule-1
EBV	Epstein-Barr-virus
ER	Endoplasmic reticulum
HAART	Highly active antiretroviral therapy
HAdV	Adenoviruses
HBV	Hepatitis B virus
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
IFN	Interferon
IL	Interleukin
ISRE	IFN-stimulated response element
ITAM	Immunoreceptor tyrosine-based activating motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
ITSM	Immunoreceptor tyrosine-based switch motif
JRC	Jurkat reporter cells
KIR	Killer cell immunoglobulin-like receptor
KO	Knock out
KSHV	Kaposi's sarcoma-associated herpesvirus
MAP	Mitogen-activated protein

MIC	MHC class I polypeptide–related sequence
NCR	Natural cytotoxicity receptor
Necl	Nectin-like
Nef	Negative regulatory factor
NFκB	Nuclear factor-κB
NK	Natural killer
NKG2	Natural killer group 2
OC	Open conformer
PVR	Poliovirus receptor
RNA	Ribonucleic acid
SPR	Surface plasmon resonance
TAP	Transporter associated with antigen processing
TIGIT	T-cell immunoreceptor with immunoglobulin and ITIM domains
TNF	Tumour necrosis factor
Vif	Viral infectivity factor
Vpr	Viral protein R
Vpu	Viral protein U
β_2m	β_2-microglobulin

Abstract

Natural killer (NK) cells are important innate effector cells contributing to the control of viral infections. Through the expression of a large array of activating and inhibitory receptors, including killer cell immunoglobulin-like receptors (KIRs), NK cells are able to sense and eliminate virus-infected cells. Persistent viral infections, such as HIV-1 and HCV infections, are difficult to control, as these viruses have evolved several mechanisms to escape immune recognition. Certain NK cell populations expressing specific KIRs have been associated with a better outcome of HIV-1 and HCV infection. For a better understanding of the underlying functional mechanisms leading to antiviral NK cell activity, we analyzed the impact of interactions between KIR2DL5 and KIR3DS1 and their respective non-classical ligands on the control of HIV-1 and HCV infection. Recently, CD155 was suggested to serve as a binding partner for KIR2DL5. However, the functional consequences of the new interaction for NK cells during viral infections are not well understood. Previous studies have described an HIV-1-mediated modulation of CD155 levels on the surface of infected cells. We confirmed binding of KIR2DL5 to CD155 and observed a reduced activity of KIR2DL5$^+$ NK cells against CD155 expressing target cells. Furthermore, we showed that HIV-1 downregulates CD155 expression on the surface of infected cells in a Nef-dependent mechanism, leading to an increased antiviral activity of KIR2DL5$^+$ NK cells against wild type compared to Nef-deficient HIV-1 strains. Gene association studies repeatedly described an association between individuals encoding for *KIR3DS1* and a better outcome of HCV infection. In our study, we demonstrated that the KIR3DS1 interaction partner HLA-F is upregulated on HCV-infected cells *in vitro* and *in vivo*. The elevated HLA-F surface levels resulted in an increased inhibition of HCV replication by KIR3DS1$^+$ NK cells and NK cells of individuals encoding for *KIR3DS1*. Our data demonstrate functional consequences of HIV-1-mediated changes of CD155 levels on infected cells, thereby modulating the antiviral activity of KIR2DL5$^+$ NK cells, and that interactions between KIR3DS1 and HLA-F contribute to NK cell-mediated control of HCV infection. Therefore, the herein described consequences of interactions between KIRs and their ligands on virus-infected cells provide new insights into the antiviral activity of KIR$^+$ NK cells during HIV-1 and HCV infection and furthermore represent promising targets for the development of future immunotherapeutic treatments.

Zusammenfassung

Natürliche Killerzellen (NK-Zellen) sind Teil des angeborenen Immunsystems und tragen zur Immunantwort gegen verschiedene virale Infektionen bei. NK-Zellen besitzen die Fähigkeit virusinfizierte Zellen zu erkennen und zu eliminieren. Dies geschieht über Interaktionen zwischen verschiedenen aktivierenden und inhibierenden NK-Zell-Rezeptoren und ihren Liganden auf der Oberfläche von anderen Zellen. Eine wichtige Gruppe von NK-Zell-Rezeptoren sind *killer cell immunoglobulin-like receptors* (KIRs). Persistierende Virusinfektionen, wie HIV- und HCV-Infektionen, lassen sich nur unzureichend vom Immunsystem kontrollieren, da die Viren Strategien entwickelt haben, um den Abwehrmechanismen des Wirts zu entgehen. In genetischen Studien wurden bestimmte KIRs mit besseren Verläufen von HIV- und HCV-Infektionen assoziiert. Um die zugrundeliegenden Mechanismen die zu einer erhöhten antiviralen Aktivität von NK-Zellen führen besser zu verstehen, wurde der Einfluss von Interaktionen der NK-Zell-Rezeptoren KIR2DL5 und KIR3DS1 mit ihren jeweiligen Liganden CD155 und HLA-F auf die Kontrolle von HIV- und HCV-Infektionen untersucht.

KIR2DL5 bindet an CD155, die funktionellen Konsequenzen der Interaktion für NK-Zellen während viraler Infektionen sind jedoch weitgehend unbekannt. Einige Studien weisen darauf hin, dass HIV-1 die Expression von CD155 auf der Oberfläche von infizierten Zellen moduliert. In dieser Arbeit wurde bestätigt, dass KIR2DL5 an CD155 bindet und gezeigt, dass die Interaktion zu einer Hemmung der antiviralen Aktivität von KIR2DL5$^+$ NK-Zellen durch CD155 exprimierende Zellen führt. Darüber hinaus wurde gezeigt, dass HIV-1 die CD155-Expression auf der Oberfläche infizierter Zellen durch einen Nef-abhängigen Mechanismus herunterreguliert, was in einer erhöhten antiviralen Aktivität von KIR2DL5$^+$ NK-Zellen gegen HIV-1 Wild Type Stämme im Vergleich zu Nef-defizienten HIV-1-Stämmen resultiert. In Genassoziationsstudien wurde wiederholt ein Zusammenhang zwischen Individuen, die für *KIR3DS1* kodieren, und einem besseren Ausgang von HCV-Infektionen beschrieben. Durch die Verwendung von *in vitro* und *in vivo* Modellen konnte demonstriert werden, dass der KIR3DS1 Interaktionspartner HLA-F vermehrt auf der Oberfläche von HCV-infizierten Zellen exprimiert wird. Die erhöhte HLA-F-Oberflächenexpression führte zu einer verstärkten Hemmung der HCV-Replikation durch KIR3DS1$^+$ NK-Zellen und NK-Zellen von Personen, die für *KIR3DS1* kodieren.

Die Ergebnisse dieser Arbeit zeigen funktionelle Konsequenzen der HIV-1-vermittelten Verringerung der CD155 Expression für die antivirale Aktivität von KIR2DL5$^+$ NK-Zellen, sowie, dass Interaktionen zwischen KIR3DS1 und HLA-F zur Kontrolle von HCV-Infektionen beitragen können. Die hier beschriebenen Auswirkungen der Interaktionen zwischen KIRs und ihren Liganden auf virusinfizierten Zellen bieten daher neue Einblicke in die antivirale

Aktivität von KIR^+ NK-Zellen während HIV-1- und HCV-Infektionen und stellen darüber hinaus mögliche Ziele für die Entwicklung künftiger immuntherapeutischer Behandlungen dar.

1 Introduction

1.1 Human immune system

The human immune system is essential for the protection of organisms against malignancies and infections with pathogens, such as bacteria, parasites, fungi and viruses, by providing the ability to distinguish between “self” and “non-self” molecules [2,3]. The different immune compartments are divided into two functional distinct parts, the innate- and the adaptive immune system [4]. The innate immune system includes physical and anatomical barriers and develops an immediate response involving a variety of myeloid and lymphoid cells, such as macrophages, granulocytes, mast cells, dendritic cells (DCs) and NK cells [5,6]. The rapid activation of the innate immune system represents the first line of defence against transformed or infected cells. In contrast, the adaptive immune system, also called the acquired immune system, develops during the lifetime of an individual and takes over if the innate immune system was not sufficient to effectively destroy the pathogen. It is mainly composed of two specialized lymphocytes, the B and T cells [7]. While innate immunity provides a more general and non-specific response against foreign invaders, the adaptive immune system develops a highly antigen specific defence after an initial contact to a pathogen, involving specific antigen receptors and the development of specific antibodies. Furthermore, its ability to create an immunological memory leads to enhanced responses and protection against reinfection at future exposures [8,9]. However, effector cells of the innate immune system are crucial for the early control of many infections and can prevent the manifestation of chronic infectious diseases [10–12].

1.2 Natural killer cells

NK cells are important effector lymphocytes of the innate immune system with the ability to recognize abnormal and infected cells without the need of prior sensitisation. They are able to distinguish between “self” and “non-self” molecules by expressing a large array of activating and inhibitory receptors [13]. NK cells play an important role in the early immune response against viral infections [14,15]. In contrast to adaptive immune cells, effector functions of NK cells are regulated through pre-expressed germline-encoded receptors, enabling an early recognition and elimination of infected host cells [16]. NK cells represent between 5-15% of peripheral blood lymphocytes and almost 50% of all lymphocytes within the liver. In addition, NK cells are also present in the gut, spleen, uterus, lung and bone marrow, and to a lower level in the thymus, secondary lymphoid tissue and mucosa-associated tissue [17–19]. NK cells are defined as $CD14^{-}/CD19^{-}/CD3^{-}/CD16^{+/-}/CD56^{+}$ and can be divided into two functional and phenotypical distanced subpopulations that are

characterized by their differential CD56 and CD16 expression levels [18]. The $CD56^{dim}$ NK cells express low levels of CD56 and high levels of CD16 and represent 90% of total circulating peripheral blood NK cells. $CD56^{dim}$ NK cells express a high variety of killer cell immunoglobulin-like receptors (KIRs) and harbour high amounts of cytolytic granular, containing perforin and granzymes, enabling a strong cytotoxic capacity. NK cells expressing high levels of CD56 and lacking the expression of CD16 are defined as $CD56^{bright}$ NK cells. This subset displays lower KIR expression levels and is present with a frequency of around 10% of peripheral blood NK cells. $CD56^{bright}$ NK cells release high amounts of cytokines, such as IFNγ, TNFa, IL-10 and IL-13 as their main effector function [18]. With the ability to detect and destroy virus-infected cells in early stages of infection, NK cells play a major role in immune defence against viral infections including infections with the human immunodeficiency virus (HIV) and hepatitis C virus (HCV).

1.2.1 Effector functions and recognition of virus-infected cells

NK cells are able to recognize and eliminate abnormal and virus-infected cells in a cell-contact-dependant manner, without being sensitised prior to exposure. NK cells contribute to the innate immune response and the control of viral infections by exhibiting cytotoxic effector functions as well as by releasing cytokines. Furthermore, NK cells shape the response of other immune cell subsets by the production of cytokines and direct cell-cell interactions [20,21].

Cytotoxic effector functions of NK cells are mediated through different mechanisms, including the induction of apoptosis in target cells by binding to death receptors [22,23] and by antibody-dependant cytotoxicity (ADCC), mediated through the FcγRIII receptor CD16 [16]. Furthermore, cytotoxicity is mediated through exocytosis of cytotoxic granules containing perforin and granzymes [24]. To implement a tolerance against healthy tissue and to prevent tissue damage, NK cells are tightly regulated by the expression of an array of activating and inhibitory receptors. These receptors and their respective ligands enable NK cells to distinguish between harmless “self” and “non-self” and determine if NK cells become activated or remain quiescent [13]. Activating NK cell receptors can detect molecules such as stress-induced self-ligands or virus-induced non-self ligands [25,26]. Under normal conditions, all nucleated healthy host cells express human leukocyte antigen (HLA) class I molecules on the cell surface, which are recognized by most inhibitory NK cell receptors [27]. Inhibitory signals overcome the strength of activating signals, which subsequently leads to inhibition and a state of “tolerance” against normal healthy cells [28,29]. In the case of an infection, viruses tend to modulate the surface proteins of infected cells and this modifications are sensed by NK cells [30]. The downregulation of surface

molecules and the presentation of viral epitopes or self-stress epitopes by HLA class I interferes with the binding of inhibitory receptors, resulting in a loss of inhibitory signals ("missing-self") [28,31]. Simultaneously, activating ligands can be upregulated and activating signals become dominant, leading to an activation of the NK cell ("induced-self") and an elimination of the target cell [32] (Figure 1).

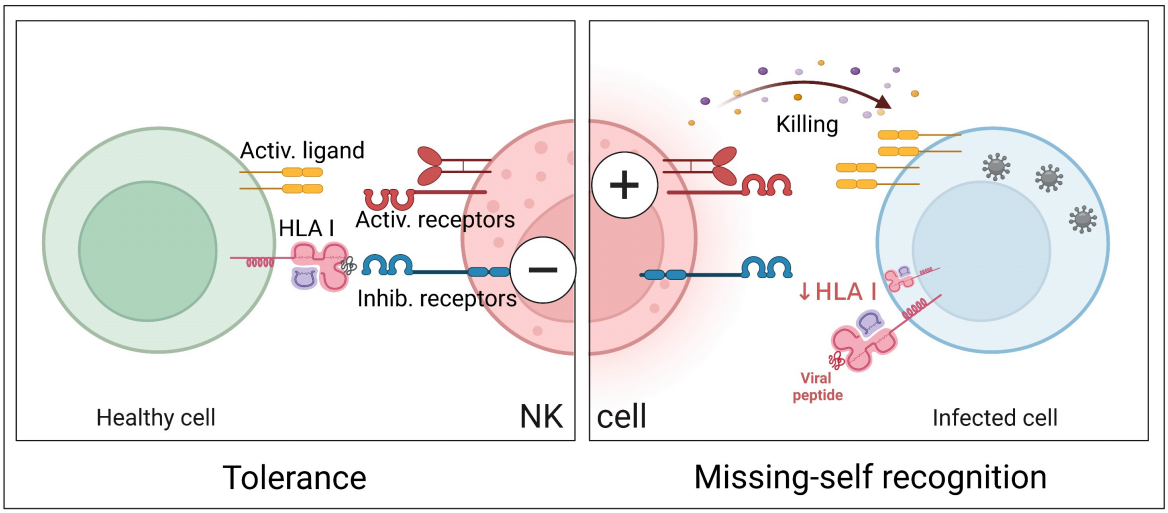

Figure 1: NK cell function. NK cell function is regulated by activating receptors binding to activating ligands and inhibitory receptors interacting mainly with HLA class I molecules on healthy cells. Under normal conditions, inhibitory signals are dominant and establish a state of "tolerance". On infected cells, HLA class I molecules are often downregulated or they present viral peptides that alter binding to NK cell receptors, and activating ligands are upregulated. This leads to a loss of inhibitory signals and increased activating signals, resulting in "missing-self recognition" and killing of infected cells.

1.2.2 NK cell receptors and their cellular ligands

NK cells express several functional and structural distinct groups of receptors that contribute to the tight regulation of NK cell effector function [16]. Beside the Fc receptor CD16, which is present on all $CD56^{dim}$ peripheral blood NK cells, NK cells express three important receptor families. (i) The mainly activating natural cytotoxicity receptors (NCRs), including NKp30, NKp44 and NKp46. (ii) The natural killer group 2 (NKG2) receptors that are C-type lectin family receptors, having either activating (e.g. NKG2C/D) or inhibitory (NKG2A) functions and (iii) the predominantly inhibitory killer cell immunoglobulin-like receptors (KIRs) (Figure 2) [13,16,33,34]. Each receptor recognizes specific ligands, which can be either self-molecules (HLA class I), stress induced self-ligands (MIC-A/B, ULBP) and non-self-ligands in the context of HLA class I or bacterial-, virus-derived and tumour cell ligands directly on the cell surface of target cells [26,34–37]. More recently, another group of NK cell receptors has emerged to be involved in balancing NK cell activity in human diseases such as cancer and infections. The nectin and nectin-like protein binding receptors CD226 (DNAM-1), T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT), CD96

(Tractile) [38] and, as described here and by others, the novel CD155 (Necl-5) binding receptor KIR2DL5 [39–41] (Figure 2).

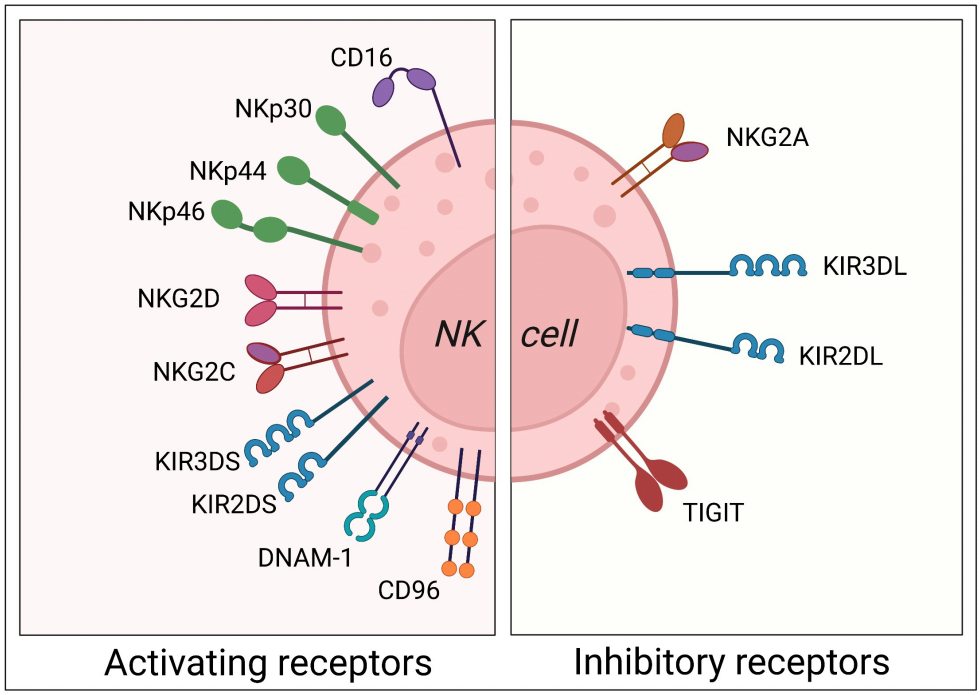

Figure 2: NK cell receptors. NK cells express a large array of activating and inhibitory receptors that upon engagement can lead to inhibition or activation of NK cell effector functions. Beside the expression of the FcγRIII receptor CD16, NK cells express several important groups of receptors including natural killer group 2 receptors (NKp30, NKp44, NKp46), C-type lectin family receptors (NKG2A, NKG2C, NKG2D), killer-cell immunoglobulin-like receptors (KIRs) and the group of nectin/nectin-like receptors (DNAM-1, TIGIT, CD96).

Interactions between inhibitory NK cell receptors such as NKG2A and inhibitory KIRs with their respective self-HLA class I ligands result in functional maturation and self-tolerance of NK cells. This mechanism, called education or licensing, results in increased responsiveness of NK cells against target cells and a retained tolerance against healthy cells. Uneducated cells lacking the expression of inhibitory receptors or the engagement of inhibitory receptors with the specific ligands remain hyporesponsive and are less effective in killing target cells [42,43].

1.2.3 Killer cell immunoglobulin-like receptors and their ligands

KIRs are a family of NK cell receptors that are involved in the tight regulation of NK cell function during health and disease. The group of type I transmembrane glycoproteins are expressed on the surface of NK cells and a subset of T cells and can mediate either activating or inhibitory signals after interacting with specific HLA class I molecules on the

surface of other cells [44]. *KIR* genes are located on chromosome 19q13.4 and encode 15 highly polymorphic genes (*KIR2DL1, KIR2DL2, KIR2DL3, KIR2DL4, KIR2DL5A, KIR2DL5B, KIR2SD1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5, KIR3DL1, KIR1DS1, KIR3DL2, KIR3DL3*), which have been divided into two haplotypes, namely A and B. Both haplotypes are present in all human populations to a similar extend. While they share the presence of several inhibitory KIR genes, they mainly differ in the number of activating KIRs. Haplotype A has one activating KIR (KIR2DS4) and haplotype B can include up to five activating KIRs [44,45]. Furthermore, the inhibitory KIR2DL5 and KIR2DL2 are exclusively assigned to haplotype B [46]. The highly polymorphic KIR alleles lead to various KIR expression patterns between individuals and the high numbers of different KIRs expressed enable sensing of changes in HLA class I expression on target cells [47]. KIRs are highly polymorphic; however, they are structurally closely related. They are type I transmembrane glycoproteins which differ in the presence of two (KIR2D) or three (KIR3D) extracellular Ig-like domains and a short (S) or a long (L) intracellular tail, providing the basis for the KIR nomenclature. KIRs are either inhibitory or activating, depending on their intracellular composition. Inhibitory KIRs have a long cytoplasmic domain containing immunoreceptor tyrosine-based inhibitory motifs (ITIMs), which recruit protein tyrosine phosphatases such as SHP1 or SHP2 upon ligand-mediated receptor crosslinking, leading to the transfer of inhibitory signals into the cell. In contrast, KIRs with short intracellular tails act as activating receptors and co-localize with adaptor molecules such as DAP12, that facilitate the activating signalling by immunoreceptor tyrosine-based activating motives (ITAMs) containing tyrosine residues that are phosphorylated upon activation [44] (Figure 3).

HLA class I molecules are grouped into classical (HLA-A, -B, -C) and non-classical (HLA-E, -F, -G) and are encoded on chromosome 6 [48]. KIRs mainly bind to classical HLA class I molecules, which are broadly expressed by almost every nucleated cell in the human body and are encoded by highly polymorphic genes. For most KIRs specific HLA class I molecules have been identified as functional ligands. KIR2DL1 and KIR2DL3 bind to HLA-C group 2 and group 1 molecules, respectively, and KIR3DL1 recognizes HLA-Bw4 molecules [45,49,50]. While most KIRs bind to classical HLA class I allotypes on the surface of normal healthy cells, a minority of KIRs also interact with non-classical KIR ligands. The activating KIR3DS1 is binding to open conformers (OCs) of HLA-F on the cell surface [51] and the structural and functional unconventional KIR2DL4 binds to HLA-G molecules [52]. KIR2DL4 contains a long intracellular tail that is associated with an ITAM-containing FcεRI-γ adaptor instead of a DAP12 protein, allowing the establishment of activating signals [53,54]. The structurally related but functional distinct inhibitory receptor KIR2DL5 is one of very few KIRs for which no HLA ligand has been identified so far. Recently, the poliovirus receptor (PVR, CD155) has been described as an interaction partner, making KIR2DL5 the first KIR

interacting with a non-HLA ligand [39–41]. More recently, KIR3DL3 has been described to interact with HHLA2, adding another KIR that binds to non-HLA molecules [55].

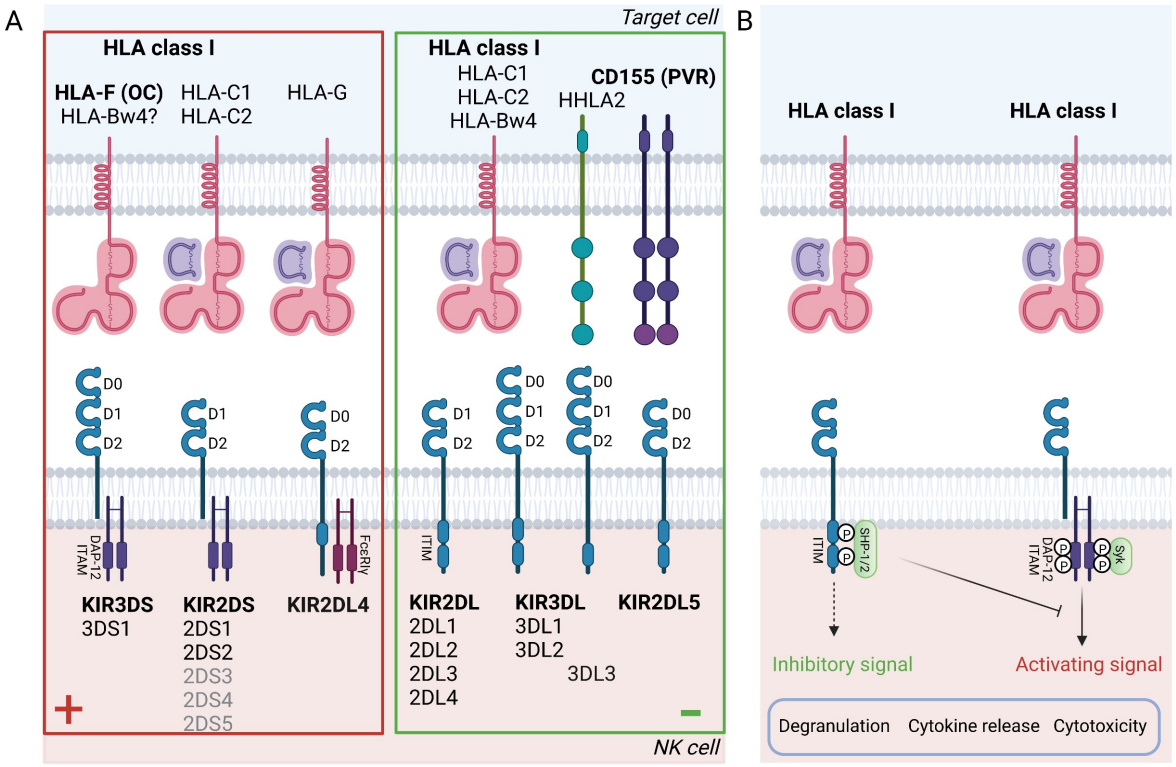

Figure 3: Interactions between KIRs and their cellular ligands. A KIRs are activating or inhibitory receptors characterized by a short cytoplasmic tail associated with an adapter protein (DAP12) containing immunoreceptor tyrosine-based activating motifs (ITAMs), or a long cytoplasmic tail with immunoreceptor tyrosine-based inhibitory motifs (ITIM), respectively. KIR2DL4 has a long intracellular tail that is associated with an ITAM-containing FcεRI-γ adaptor, resulting in activating functions. KIRs further differ in their extracellular part by containing two or three extracellular domains. Classical KIR ligands are HLA class I molecules on the surface of other cells. KIR3DL3 and KIR2DL5 bind to the non-classical ligands HHLA2 and CD155, respectively. KIRs for which no ligands have been identified so far are marked in gray. **B** Signaling of activating KIRs in response to receptor crosslinking is mediated by phosphorylation of tyrosine residues within the ITAMs and a recruitment of Syk tyrosine kinases. Inhibitory signals are established by tyrosine phosphorylation of ITIMs and a recruitment of tyrosine phosphatases (SHP-1/2). KIR2DL4 represents an exception, as the receptor is associated with an ITAM containing adapter protein FcεRI-γ enabling the establishment of activating signals.

1.2.3.1 KIR2DL5/CD155

KIR2DL5 (CD158f) is the most recently described KIR that is expressed by human T cells and NK cells [56]. KIR2DL5 was known as an orphan receptor with no identified ligands for a long time [57]. While the receptor shares similar structural patterns with other KIRs, its genetics are complicated by a duplication of the gene that is exclusively found in humans [58,59]. Like other KIRs, KIR2DL5 is genetically highly polymorphic and the 92 identified alleles (The Immuno Polymorphism Database, as of July 2022 [60]) are encoded by two

different loci, designated as *KIR2DL5A* and *KIR2DL5B*, located on the telomeric and the centromeric part on chromosome 19 in the KIR locus, respectively [59]. *KIR2DL5* genes are controlled by three structurally different promotor types, which, together with epigenetic modifications, define transcription and expression of the *KIR2DL5* alleles [61]. While transcribed alleles are controlled by type I or type III promotors, silent KIR2DL5 genes are controlled by type II promotors [57,62]. In 2007, Estefania et al. generated the first monoclonal antibody directed against KIR2DL5 [63]. However, surface expression was mainly detected for molecules encoded by *KIR2DL5A*, in particular KIR2DL5A*001 [63]. It remains elusive if the KIR2DL5 antibody clone UP-R1 can only detect this allotype specifically or if other allotypes are not expressed on the cell surface. *KIR2DL5* alleles exist in all ethnic populations with frequencies ranging between 26% and 86% and different contributions of the paralogues *KIR2DL5A* and *KIR2DL5B.* KIR2DL5 molecules are expressed by up to 15% of peripheral blood NK cells in healthy individuals encoding for KIR2DL5A*001 [57]. KIR2DL5 has a typical structure with two Ig-like domains of the D0-D2 type, which can also be found in the structurally closely related KIR2DL4 [56]. While KIR2DL4 binds to HLA-G [52], ligands for KIR2DL5 remained unknown for more than two decades. Therefore, the functional relevance of KIR2DL5 in human health and disease was not well understood. The cytoplasmic tail of KIR2DL5 contains a typical ITIM and an atypical ITIM, similar to an immunoreceptor tyrosine-based switch motif (ITSM) that is unique in the family of human KIRs [56]. Antibody crosslinking of naturally expressed KIR2DL5 led to inhibition of NK cell cytotoxicity [63]. Experiments using KIR2DL5^{+} NK cells demonstrated a SHP-2-dependant signalling upon receptor phosphorylation [64]. Recently, the nectin-like molecule CD155 (Necl-5, PVR) was described as a binding partner for KIR2DL5 by performing proteomic screenings [39,40], identifying one of the first KIRs that does interact with other proteins than HLA class I molecules. Additionally, heparan sulfate proteoglycans have been identified as binding partners for several KIRs containing a D0 domain, including KIR2DL5 [65].

CD155 is a nectin-like molecule, also known as Necl-5, and is encoded by the *poliovirus receptor (PVR)* gene. Nectin and nectin-like molecules are members of the immunoglobulin superfamily, playing an important role in cell adhesion, cell movement, proliferation and survival [66,67]. A group of Nectin and nectin-like molecules has recently been identified as important regulator of NK cell function by interacting with the NK cell receptors DNAM-1, TIGIT and CD96 [38] (Figure 4). The activating DNAM-1 was initially described as an adhesion molecule that is involved in the control of NK cell-mediated cytotoxicity and is intensively studied. DNAM-1 is expressed by most resting NK cells and its ligands are CD112 (Nectin-2) and CD155 (PVR) [68]. Both, CD112 and CD155 are also recognized by

the inhibitory receptor TIGIT, while the inhibitory and/or activating CD96 interacts with CD111 (Nectin-1), CD112 and CD155 [69–71], revealing a highly balanced system impacting NK cell function. CD155 is expressed in most adult organs at low levels and to a higher degree in regenerating liver tissues and on antigen-presenting cells (APCs) [72,73]. CD155 expression is regulated by cellular stress and is often upregulated in the setting of transformed cells and during viral infections [72,74,75]. Overexpression of CD155 in tumors is associated with enhanced tumor cell movement and proliferation, making it an unfavourable tumor marker as an indicator for cancer invasiveness and metastasis [76]. CD155 upregulation is mediated through several signalling pathways, including the mitogen-activated protein (MAP) kinase and nuclear factor-κB (NFκB) pathways and the DNA damage responses (DDR) [72,74,77,78]. The later has especially been associated with changes in CD155 expression during viral infections, as the DNA damage sensors can detect foreign genetic material and function as a cell intrinsic immune pathway [79–81]. Furthermore, CD155 expression is upregulated on APCs in response to toll-like receptor activation [72]. In general, CD155 has an immune stimulating effect, especially by interacting with the activating receptor DNAM-1. Elevated CD155 expression levels on virus-infected cells potentially trigger DNAM-1-mediated NK cell recognition. Some viruses have evolved mechanisms to impair CD155 expression [82,83], potentially to counteract DNAM-1-dependant NK cell activation, further highlighting the importance of the interaction for the control of viral infections. Recently, the inhibitory KIR2DL5 has been described to also interact with CD155 [39,40], adding another NK cell receptor to this axis.

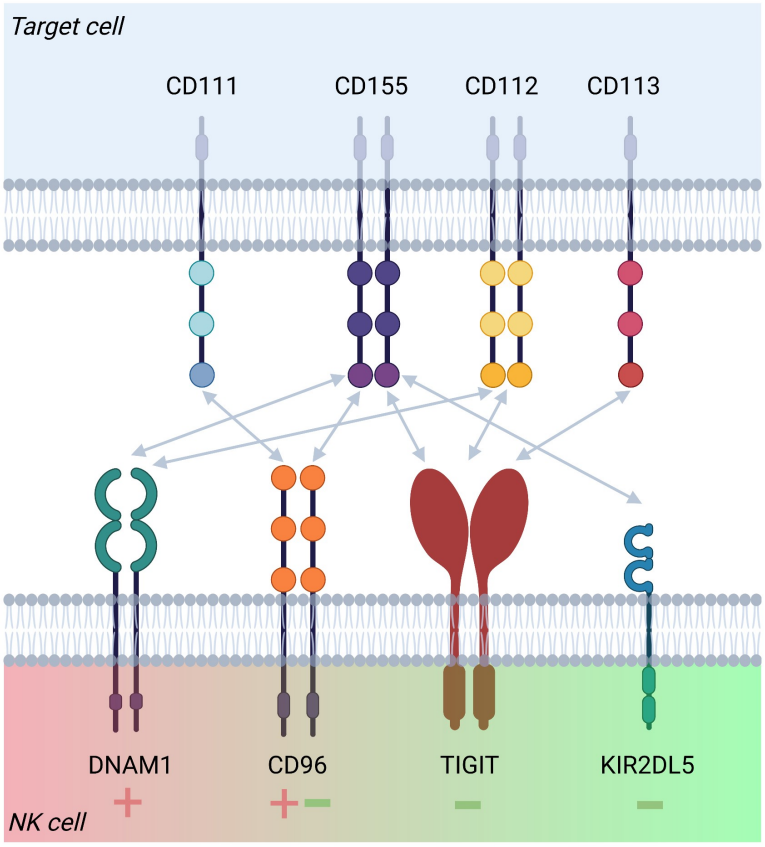

Figure 4: Interactions between NK cell receptors and nectin/nectin-like molecules. Nectin and nectin-like molecules contribute to the tight regulation of NK cell functions by interacting with inhibitory and activating NK cell receptors. The activating DNAM-1 receptor interacts with CD155 and CD112. CD96 expressed on NK cells can mediate both, activating or inhibitory signals after binding to CD111 and CD155. The inhibitory TIGIT receptor is a binding partner of CD155, CD112 and CD113, and KIR2DL5 binds to CD155 on the surface of other cells.

1.2.3.2 KIR3DS1/HLA-F

The activating KIR3DS1 is associated with the outcome of several human diseases [47,84]. The *KIR3DS1* gene is present with varying frequencies in all human populations and is encoded in the *KIR3DL1/S1* gene locus [85]. In comparison to most other KIRs, KIR3DS1 represents a less polymorphic variant [85] with 39 identified alleles in humans (The Immuno Polymorphism Database, as of July 2022 [60]). The frequency of KIR3DS1-expressing NK cells in one individual can range between 10-80%, also depending on homo- or heterozygosity of the KIR3DS1 allele [84]. While KIR3DL1 and KIR3DS1 share the same gene locus as well as a very similar extracellular domain, they differ fundamentally in functionality and ligand specificity. In contrast to the inhibitory KIR3DL1, KIR3DS1 is an activating receptor, mediating NK cell cytotoxicity and IFNγ production [86]. While KIR3DS1 represents the only activating KIR with three extracellular domains, it contains a typical short cytoplasmic tail that allows recruitment of DAP12 adaptor molecules establishing activating

signals. Ligands for KIR3DL1 are well established, including the classical HLA class I molecules HLA-A and HLA-B with a Bw4 motif [49,87]. However, for KIR3DS1 specific ligands remained unknown, except one study describing a peptide-dependent binding of KIR3DS1 to HLA-B*57:01 [88]. Genetic association studies repeatedly highlighted a correlation of *KIR3DS1* and the disease outcome of HIV-1 infection in patients encoding for HLA-Bw4 [89,90], however, direct interactions between KIR3DS1 and HLA-Bw4 have not been described. Furthermore, KIR3DS1 has been linked to different outcomes of several other viral infections [51,91–95] as well as malignancies [96,97]. In 2018, Garcia-Beltran et al. showed a high affinity interaction of KIR3DS1 with open conformers (OCs) of the non-classical HLA-F molecule, leading to degranulation of KIR3DS1$^+$ NK cells and production of antiviral cytokines [51].

HLA-F is less polymorphic compared to other HLA class I molecules and also differs in expression patterns [98]. While classical HLA class I molecules are broadly expressed on most cell types, HLA-F is described as a stress-induced self-molecule being upregulated on activated and virus-infected cells [51,93,99,100]. HLA class I complexes consist of a membrane-bound heavy chain, including three extracellular domains (α1/2/3) and an associated β_2-microglobulin (β_2m). The extracellular domains α1 and α2 form a binding groove, loaded with antigenic peptides (Figure 5) [101]. HLA class I molecules can also exist in a second structural form known as HLA class I OCs, consisting of the heavy chain without associated β_2m or a bound peptide [102] (Figure 5). Several studies have suggested that HLA-F is dominantly expressed as OCs in response to cellular stress [103,104]. In comparison to other HLA class I molecules that are rather unstable as OCs, HLA-F OCs appear to be more stable [99,105]. Initially, HLA-F was one of the least studied HLA class I molecules, as the clinical relevance and biological function was poorly understood. In recent years, several studies have revealed an important function of HLA-F in combination with its newly discovered interaction partner KIR3DS1 in several infectious diseases [51,93–95,99,106].

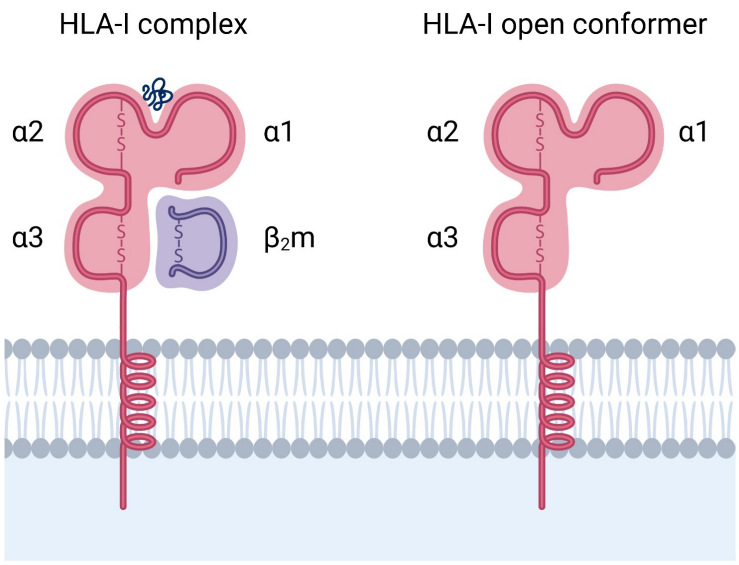

Figure 5: Conformations of HLA class I molecules. Most KIRs bind to HLA class I molecules (HLA-A/-B/-C,-E,-G), which are mainly presented as complexes consisting of a membrane bound heavy-chain (α1/2/3) associated with β_2m and loaded with a peptide. HLA class I open conformers consists of only the alpha-heavy chain without β_2m and peptide.

1.3 KIRs in viral infections

KIRs are involved in the tight regulation of the antiviral NK cell activity and contribute to the recognition of activated, stressed and infected cells [47]. The outcome of viral infections varies between individuals. Specific host genetic factors have been associated with different courses of viral diseases, including delayed progression to HIV-caused acquired immunodeficiency syndrome (AIDS) and a better control and increased rate of spontaneous clearance of HCV infection [107]. The KIR and HLA loci represent the most polymorphic regions within the human genome and allelic profiles are highly variable between individuals. Specific KIR-HLA combinations have been correlated with different outcomes of infectious diseases, autoimmunity and inflammatory disorders [108].

Little is known about the impact of *KIR2DL5* in the context of viral infections. A few studies suggest *KIR2DL5* to be beneficial for the course of HIV-1 infection [109,110], while others show a poor outcome of infection of HIV-1 and HCV infection [111,112]. A more intensively studied KIR in the context of viral infections is *KIR3DS1*. In several gene association studies, *KIR3DS1* has been associated with a protective effect against HIV-1 and HCV infection [91,113]. In combination with HLA-Bw4 it has been associated with spontaneous clearance of HCV infection [114] as well as with slower progression to AIDS [89,90]. More recent *in vitro* studies showed that KIR3DS1$^+$ NK cells contribute to the control of HIV-1, HCV, HAdV and BKPyV infection by interacting with HLA-F on the surface of infected cells [51,93–95]. Furthermore, *KIR2DL3* together with its ligand *HLA-C1* was identified to be

associated with a protective effect and better clearance of HCV infection [114,115], while the combination of *KIR2DS3/HLA-C2* was identified to be a risk factor for developing chronic HCV infection [116]. Taking together, increasing numbers of studies observing disease associations implicate an important impact of KIR/ligand interactions for the outcome of HIV-1 and HCV infection.

1.4 Persistent viral infections

Viruses are small obligate intracellular infectious agents that can only replicate in living cells of a host organism such as humans. Persistent infections are caused by viruses establishing a latent or chronic infection after an early acute phase of the disease. These viruses are not cleared by infected individuals and remain in specific host cells. Among others, HIV, causing AIDS, and HCV, leading to chronic hepatitis C, are able to lead to persistent infection (Figure 6). Additional viruses causing chronic infections are adenoviruses (AdV), BK polyomavirus (BKPyV), hepatitis B virus (HBV) and the human cytomegalovirus (HCMV) [117].

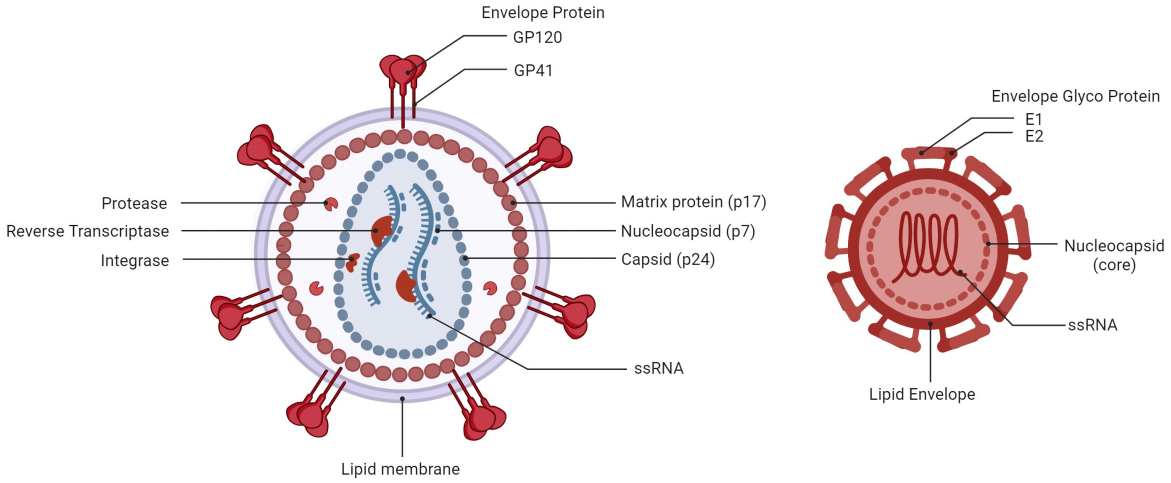

Figure 6: Structure of the human immunodeficiency virus (HIV, left) and the hepatitis C virus (HCV, right). Both viruses are enveloped by a lipid membrane harbouring glycoproteins and contain a single-stranded RNA genome, which is associated with a nucleocapsid. The HIV genome is furthermore surrounded by a capsid and a matrix and encodes for several structural and accessory proteins as well as viral enzymes. The HCV genome encodes a polyprotein that is cleaved into structural and non-structural proteins.

1.4.1 Human immunodeficiency virus

HIV is a lentivirus and belongs to the family of retroviruses. HIV originally derived from non-human primates, has spread to human populations approximately in the late 19th or early 20th century and was first described in 1983 [118–120]. Left untreated, HIV infection causes

a chronic infection ultimately leading to AIDS. In the end of 2020, over 37 million people were living with HIV and about 680.000 people died from HIV-related causes (WHO, as of June 2021 [121]), highlighting that HIV infection remains a major global health issue.

HIV mainly infects human immune cells that express the CD4 receptor, which primarily are $CD4^+$ T lymphocytes, but also monocytes, macrophages and DCs [72]. Viral particles can be detected in several body fluids, such as blood, sperm, vaginal fluid and breast milk, leading to transmission of the virus mainly through sexual contact, mother-to-child transmission or through intravenous drug usage [122]. The course of infection can be divided in different phases. After an incubation time of about three weeks, the acute phase of the infection occurs, which is marked by flu-like symptoms and a high viral load together with a first decrease in $CD4^+$ T cell numbers. Following the acute phase, HIV establishes the phase of clinical latency that is characterized by mild to no symptoms and a very low to not detectable viral loads in the blood plasma. During this phase, the $CD4^+$ T cell counts drop below a critical level, initiating the development of AIDS [119]. Due to the massive destruction of immune cells, the immune systems becomes weakened and the infected individuals are more susceptible to opportunistic infections and AIDS-related cancer [123,124].

Antiretroviral therapy (ART) can slow down the spread of the virus and the development of AIDS. Since 1996, the highly active antiretroviral therapy (HAART) was introduced, which is a combination drug therapy targeting the viral life cycle. HAART can prevent AIDS almost completely and viral loads are decreased to undetectable levels, resulting in a diminished risk for further transmission [125]. Life expectancy and quality has improved strongly for people living with HIV under HAART. However, the lifelong treatment causes several side effects, such as metabolic diseases and liver and kidney dysfunction [126–128]. Furthermore, there is limited access to HAART for HIV-positive individuals living in developing countries and the high mutation rates of the virus harbour the risk of developing viral resistances to the therapy [129]. Taking this together, there is still a need to further unravel cellular and immunological mechanisms in HIV infection to find new targets for novel therapy approaches to treat HIV infection that can overcome current limitations of HAART.

HIV is phylogenetically divided into two types, namely HIV-1 and HIV-2, which are further divided in several subtypes. Most HIV infections are caused by HIV-1 subtype M that has a high virulence and infectivity. Subtype M is further divided in nine genetically distinguished clades (A, B, C, D, E, F, G, H, J and K), which have a different prevalence around the world [119]. In North America and Europe, HIV-1 M clade B is the most common and most frequently studied subtype [130]. HIV is a single-stranded enveloped RNA virus that is reverse transcribed into double-stranded DNA in the host cell. The viral reverse transcriptase is highly error-prone, introducing a high rate of mutations into the viral

genome. Due to this high mutation rate, HIV is able to effectively escape the host immune response and to develop drug resistances [131,132]. Furthermore, viral DNA is integrated into the host cell genome, allowing the persistence of HIV in long-living resting T cells in a transcriptionally silent state [133]. These latently infected cells cannot be targeted by the currently common ARTs [134]. The HIV-1 genome is composed of nine genes including three genes encoding structural proteins and enzymes (*gag, env* and *pol*) and six genes encoding for accessory and regulatory proteins (*vif, vpu, vpr, nef, tat*, and *rev*). The highly conserved *gag* encodes for the matrix protein (p17), the nucleocapsid (p7) and the capsid (p24). *Env* is highly variable and encodes for the glycoproteins GP120 and GP41, which are involved in the attachment and the fusion of the particle with the target cell membrane. *Pol* encodes the viral enzymes (reverse transcriptase, protease and integrase) essential for the viral replication cycle. *Tat* and *rev* encode for proteins regulating viral transcription and protein expression. The accessory proteins encoded by *vif, vpu, vpr and nef* are indicated to mainly contribute to HIV infection by targeting cellular proteins to either redirect normal functions of the host protein or to inhibit the normal function of host factors by degradation or intracellular mislocalization [119,135]. Vif is critical for the infectivity of the viral particles and the multifunctional Vpr enhances viral replication. The viral protein u (Vpu) is involved in the efficient release of viral particles and facilitates degradation of CD4. Furthermore, vpu is involved in the downregulation of HLA-C and the antiviral factor tetherin from the surface of infected cells. Another protein important for viral fitness and remodelling of the host cell is the negative regulating factor (Nef). Nef is a membrane-associated protein expressed early in infection and mediates the downregulation of CD4, HLA-A, HLA-B and potentially CD155 surface expression to inhibit immune responses preventing recognition and killing of infected cells by HIV-specific $CD8^+$ T cells and NK cells [41,82,135,136].

1.4.2 Hepatitis C virus

The 1989 identified HCV belongs to the family of flaviviridae and causes one of the most common infectious diseases. Globally, about 58 million people are living with chronic HCV infection and in 2019 an estimated 290.000 people died from HCV (WHO, as of June 2022 [137]). HCV infection often results in chronic hepatitis, leading to liver cirrhosis, liver cancer and ultimately the need for liver transplantation [138,139]. While most HCV-infected individuals develop a chronic infection, approximately 20% of the patients resolve acute infection in its early stage [138]. HCV transmission mainly occurs through blood-to-blood contact and primarily through parental exposure, contaminated blood products, medical equipment and intravenous drug use. In contrast to HIV-1, sexual transmission of HCV is reported to a lesser extent [140]. The primary targets of HCV are human hepatocytes [141].

The hepatotropic virus is non-cytopathic; however, during infection constant immune activation leads to severe liver damage [142]. In the acute phase of infection, viral load increases rapidly and the first line of response is driven by the innate immune system, including type I and type III interferon production by infected cells and immune cells like macrophages and DCs, leading to activation and regulation of other immune cells such as NK cells [143]. In general, NK cells are important effector cells during the acute phase of infection, as they are highly activated and show increased cytotoxicity and production of IFNγ [12]. Therefore, NK cells play an important role in preventing the establishment of chronic infection.

The development of novel therapy approaches using direct antiviral agents (DAA) enables a better clinical outcome in HCV infection with sustained virological responses of over 95% [144]. In contrast to previous treatments with Pegylated-Interferon-α and Ribavirin, DAA specifically target HCV-encoded proteins, resulting in cure from HCV infection [145].

HCV is an enveloped RNA virus, harbouring a single-stranded viral RNA that is surrounded by a nucleocapsid (core) and an envelope carrying the glycoproteins E1 and E2 (Figure 5). The viral genome encodes for a polyprotein that is cleaved into the structural proteins (Core, E1 and E2) and several non-structural proteins (NS1, NS2, NS4A, NS4B, NS5A, NS5B) including viral proteases, an RNA helicase and a RNA-dependent RNA polymerase [146]. Due to a high degree of sequence variations, the structural proteins are involved in different immune evasion mechanisms of HCV. The envelope is targeted by neutralizing antibodies and can, due to mutations in the expressing genes, facilitate the development of a persistent infection [147]. The non-structural proteins are essential for viral replication and represent the major target for DAA therapy [148]. The lack of proof-reading function of the RNA-dependent RNA polymerase results in a high error rate, making the HCV genome highly variable and leading to the existence of several genotypes. Today, seven genotypes (1a, 1b, 2, 3, 4, 5, 6) and hundreds of subtypes are known. In Europe, the most prevalent variant is genotype 1. In general, genotype 1, 2, 4 and 5 are endemic in Africa, while the genotypes 3 and 6 are most common in Asia [149]. The high degree of variability, especially in the structural proteins, leads to the ability of HCV to adapt to the host immune system and to develop resistances to antiviral drugs.

2 Hypothesis and aims of the project

NK cells play an important role in regulating a coordinated immune control during viral infections and are associated with the outcomes of HIV-1 and HCV infection. In this thesis, I studied the antiviral activity of different NK cell populations in HIV-1 and HCV infection, especially with regards to KIR2DL5$^+$ and KIR3DS1$^+$ NK cells. To address a potential antiviral effect of these NK cell subsets, this thesis focused on two aims:

Aim 1: The validation of novel KIR2DL5 ligands and the assessment of functional consequences of interactions between KIR2DL5 and CD155 for KIR2DL5$^+$ NK cells.

Aim 2: Investigating changes in KIR ligand surface expression levels during viral infections in order to unravel the mechanisms involved in antiviral activity of KIR2DL5 and KIR3DS1 expressing NK cells during HIV-1 and HCV infection.

It was previously suggested that HIV-1 is able to modulate CD155 expression levels on the surface of infected cells [80,82,150,151]. Furthermore, genetic studies showed an association between individuals encoding for *KIR3DS1* and a better outcome in HCV infection [91,96,114]. Therefore, the following hypotheses were tested:

Hypothesis 1: Interactions between KIR2DL5 and CD155 lead to reduced antiviral activity of KIR2DL5$^+$ NK cells. HIV-1 downregulates CD155 to escape DNAM-1-dependent NK cell recognition, resulting in increased antiviral activity of KIR2DL5$^+$ NK cells.

Hypothesis 2: HCV infection induces an upregulation of the KIR3DS1 ligand HLA-F, leading to an increased viral inhibition by KIR3DS1$^+$ NK cells.

3 Discussion

NK cells are part of the innate immune system and play an important role in the early control of HIV-1 and HCV infection, enabling a fast response during the early stage of infection that can result in slower disease progression or prevention from the establishment of a chronic infection. During persistent viral infections caused by viruses such as HIV-1 and HCV, the host and virus are in a constant conflict between developing a sufficient antiviral immune response and the development of viral immune escape mechanisms [107]. Viruses tend to modulate the expression of surface molecules on infected cells, primarily to avoid immune recognition by cells of the innate and adaptive immune system. At the same time, infected cells alter their surface protein expression pattern by virus independent cell intrinsic mechanisms in response to viral infections. However, these changes are often sensed by specific immune cell subsets. Several NK cell populations, characterized by the expression of specific receptors such as KIRs, have been associated with a better control of viral replication and a better outcome in HIV-1 and HCV infection [47]. In this thesis, I aimed to assess the impact of viral infection-caused changes of surface levels of novel ligands for KIRs on the antiviral activity of different KIR^+ NK cell populations. While most KIRs involved in antiviral activity of NK cells bind to classical HLA class I molecules, we hypothesized that KIRs binding to non-classical ligands, such as CD155 and HLA-F, display an additional important group of receptors contributing to the antiviral activity of NK cells.

The focus of the thesis was (i) to assess the antiviral potential of $KIR2DL5^+$ NK cells during HIV-1 infection regarding interactions of KIR2DL5 with the novel ligand CD155, and (ii) how interactions between KIR3DS1 and HLA-F contribute to the control of HCV replication. In study #1, we confirmed binding of KIR2DL5 to CD155 by performing a bead-based ligand screening and by using KIR2DL5 reporter cell lines. Furthermore, we investigated the functional consequences of the interaction and observed that effector functions of NK cells expressing KIR2DL5 are significantly inhibited by target cells expressing CD155, leading to decreased degranulation of $KIR2DL5^+$ NK cells. HIV-1 significantly downregulated CD155 on the surface of infected cells using the viral protein Nef, resulting in increased antiviral activity of $KIR2DL5^+$ NK cells and a decreased HIV-1 replication in primary human $CD4^+$ T cells *in vitro* [41].

In study #2, we found that the KIR3DS1 ligand HLA-F is upregulated on HCV-infected cells. The upregulation was observed *in vitro* by infecting the hepatoma cell line Huh7 with HCV as well as by using a chimeric humanized mouse models and primary liver tissue samples from HCV-infected individuals as *in vivo* models for HCV infection. Given the elevated HLA-F expression on HCV-infected cells, the role of KIR3DS1/HLA-F interactions in

controlling HCV replication was investigated. We observed stronger inhibition of HCV replication by KIR3DS1^{+} compared to KIR3DS1^{-} NK cells, providing a potential mechanism behind previous observations reporting a genetic association between *KIR3DS1* and spontaneous viral clearance in HCV-infected patients [91,94].

3.1 Impact of KIR2DL5-CD155 interactions on NK cell activity

The NK cell receptor KIR2DL5 was known as orphan receptor [57] until recently CD155 has been described as ligand [39,40]. Due to the lack of known interaction partners, the functional impact of KIR2DL5 for NK cells was not well understood. As KIR2DL5 shares a similar structure with other inhibitory KIRs, including the expression of an intracellular ITIM domain, KIR2DL5 was suggested to also contribute to inhibition of NK cells [57]. Experiments using KIR2DL5-transduced NK92 cells and primary NK cells revealed an inhibitory function by crosslinking the receptor with a KIR2DL5 specific antibody, leading to a comparable inhibition observed for KIR3DL1 [63]. Binding of KIR2DL5-Fc fusion proteins to several cell lines expressing different HLA class I allotypes resulted in broad binding, suggesting an HLA-independent interaction of KIR2DL5 with target cells [57]. This observation was supported by a study showing increased KIR2DL5 expression in a TAP-deficient patient [152]. TAP-deficiency leads to a loss of HLA class I surface expression [153], supporting the idea of a non-HLA ligand for KIR2DL5. Recently, it has been shown that several KIRs containing a D0 domain in the extracellular tail do interact with heparan sulfate, including KIR2DL5 [65]. However, functional consequences of the interaction for NK cells remain unknown. The interaction with heparan sulfate might explain a broad binding of KIR2DL5-Fc fusion constructs to several cell lines that we and others observed [57]. Pre-incubation of cell lines with a sulfate-free sulfation inhibition medium, that results in the absence of heparan sulfate on the cell surface as described by Klein et al. [65], led to an abrogation of the observed binding. In Figure 7, binding of KIR2DL5-Fc constructs to K562 cells that barely express CD155 on the cell surface is shown after cells were cultured in normal- or sulfate-free sulfation inhibition medium.

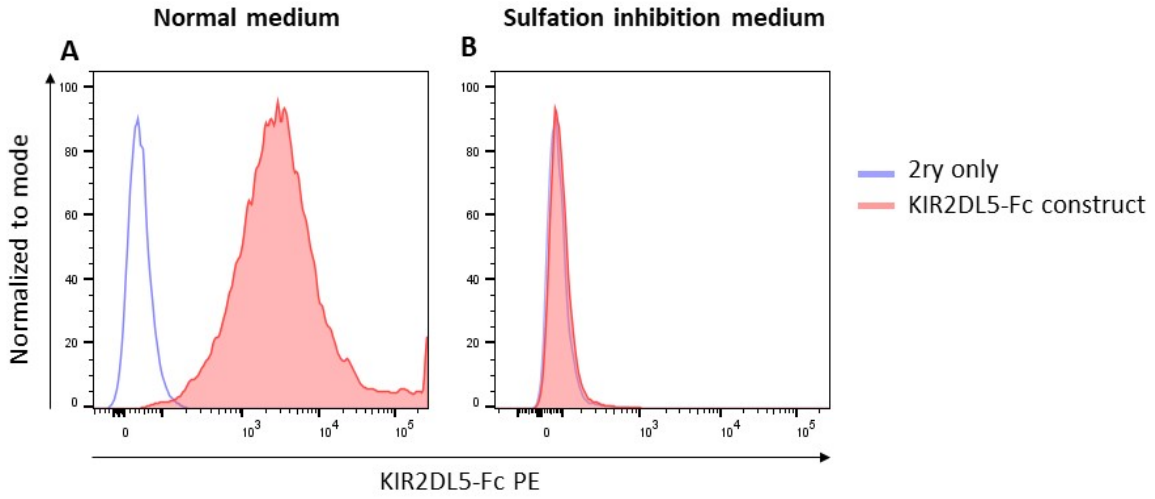

Figure 7: KIR2DL5-Fc construct binding to K562 cells with and without pre-incubation of the cells in sulfate-free sulfation inhibition medium. A K562 cells were incubated in normal cell culture medium and were stained with KIR2DL5-Fc constructs and a secondary IgG antibody (2ry) (red) or only with the 2ry antibody as a control (blue). **B** K562 cells were pre-incubated in sulfate-free sulfation inhibition medium to reduce heparan sulfate levels on the cell surface. Subsequently, cells were stained as described before.

Recently, the nectin-like molecule CD155 was described to be a binding partner of KIR2DL5 in a broad proteomic ligand screening [39], identifying the first KIR interacting with other molecules than HLA class I. Afterwards, the inhibitory KIR3DL3 has been shown to interact with HHLA2 [154], a molecule belonging the B7 protein family, adding a second KIR that binds to non-HLA ligands. The identification of non-HLA ligands for KIRs adds additional players to the complex regulation of NK cell functions, which so far have not been considered in most studies that investigated KIR-dependent NK cell function in health and disease.

During our studies, we showed binding of KIR2DL5 to CD155 and strong inhibition of KIR2DL5^{+} NK cells upon CD155 binding [41]. As most other KIRs interact with HLA class I molecules, we additionally assessed binding of KIR2DL5 to HLA class I molecules. While KIR2DL3 showed strong interactions with its known ligands of the HLA-C1 family, KIR2DL5 did not strongly bind to any of the tested HLA molecules. However, one could interpret a weak interaction of KIR2DL5-Fc fusion proteins with some proteins of the HLA-C2 group. More specifically, we observed a possible slight interaction of KIR2DL5-Fc with HLA-C*06:02. However, compared to our positive control using KIR2DL3-Fc, the signal was very low. We conducted additional experiments to assess potential binding of KIR2DL5 to HLA-C*06:02, which showed the highest signal among all tested HLA class I molecules. We did not observe any interaction or functional activation (CD69 expression) in those follow-up experiments using KIR2DL5-expressing Jurkat reporter cells (KIR2DL5 JRC) and HLA-C*06:02-transduced 721.221 cells compared to the controls (Figure 8). We therefore

conclude that the minimal binding observed in the KIR2DL5-Fc ligand screening assays does not represent a biologically relevant interaction. However, additional experiments and a higher sample size are required to further elaborate potential interactions of KIR2DL5 with HLA class I molecules.

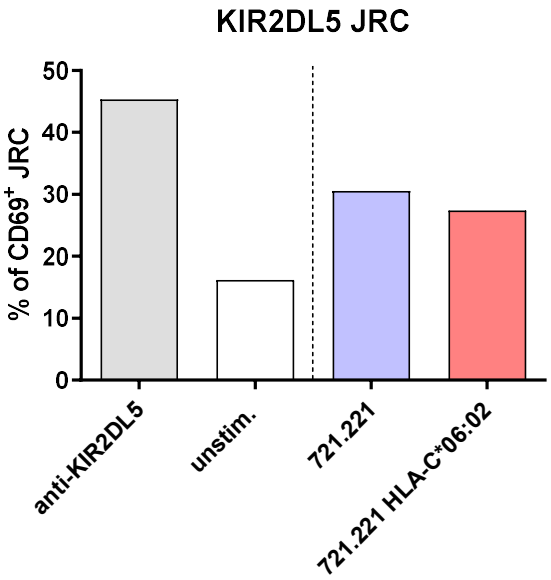

Figure 8: KIR2DL5 reporter cells are not activated upon co-cultivation with HLA-C*06:02-expressing 721.221 cells. KIR2DL5-expressing Jurkat reporter cells (KIR2DL5 JRC) were co-cultivated with anti-KIR2DL5-coated beads (positive control, gray), untransduced 721.221 cells (blue) and HLA-C*06:02-transduced 721.221 cells (red) or were left unstimulated (unstim., white). JRCs expressed a chimeric protein consisting of the extracellular domain of KIR2DL5 that was fused to an intracellular CD3ζ chain. Activation of the JRCs was determined by CD69 upregulation that served as a readout for KIR2DL5 crosslinking. Graph shows values from one experiment (*n=1*).

However, a potential interaction of KIR2DL5 with HLA class I molecules could be modulated or dependent on peptides presented by the HLA class I molecules. Several studies have shown an influence of peptide loading on the binding affinity of KIRs to their respective HLA ligands [155–158]. The peptide repertoire presented by HLA molecules can vary between self-peptides and pathogen-induced or non-self-peptides [159]. Several viruses have developed mechanisms to mimic self-peptides of the host cell or to produce viral peptides that alter binding of NK receptors. For example, it was shown that presented HIV-1 epitopes can modulate binding of inhibitory KIRs, leading to escape from NK cell recognition. HLA-Cw*01:02-restricted HIV-1 p24 epitopes and variants presented by HLA-C*03:04 were sufficient to stabilize HLA-C and were recognized by KIR2DL2 leading to inhibition of KIR2DL2$^+$ NK cells [156,157]. Furthermore, HLA-C*03:04-restricted HIV-1 p24 Gag sequence variants are associated with viral escape from KIR2DL3$^+$ NK cells [158]. Also virus-independent peptides have been shown to modulate KIR binding, such as peptide-

specific engagement of the activating NK cell receptor KIR2DS1 [155]. Altogether, it might be possible that KIR2DL5 binds to HLA molecules under specific conditions.

NK cell effector function is strongly regulated by activating and inhibitory NK cell receptors and among them, a group of receptors bind to molecules of the nectin and nectin-like family [38]. The interaction between KIR2DL5 and CD155 adds another axis to the complex regulation of NK cells by molecules of the nectin and nectin-like family, raising evidence that these interactions play an important role for NK cell function also during the course of infections. CD155 is often referred to as an NK cell activating ligand due to interactions with the activating receptor DNAM-1 [38,82]. However, two additional NK cell receptors that bind to CD155, namely CD96 and TIGIT possess inhibitory motives counterbalancing DNAM-1-mediated activation of NK cells [38]. Functional consequences of interactions between CD96 and its ligands are not fully understood, as CD96 potentially can mediate inhibitory and activating signals [71]. DNAM-1 is expressed by most human peripheral blood NK cells [160] and is therefore co-expressed with KIR2DL5 (Figure 9). We observed that KIR2DL5-CD155-mediated inhibitory signals overcome possible activating signals introduced through DNAM-1, as degranulation (CD107a expression) of primary KIR2DL5$^+$ NK cells in response to CD155$^+$ target cells decreased compared to CD155$^-$ target cells (Figure 9). Furthermore, TIGIT expression is comparable between KIR2DL5$^+$ and KIR2DL5$^-$ NK cells. However, only KIR2DL5$^+$ NK cells showed decreased degranulation against CD155$^+$ target cells, while TIGIT expression alone seems not to be sufficient to completely inhibit KIR2DL5$^-$ NK cells (Figure 9). Of note, in our study we observed similar decrease in degranulation, when we incubated KIR2DL5$^+$ NK cells with CD155-transduced compared to untransduced 721.221 target cells [41]. This observation indicates an important impact of KIR2DL5 for NK cell function and missing-self recognition of target cells by KIR2DL5$^+$ NK cells.

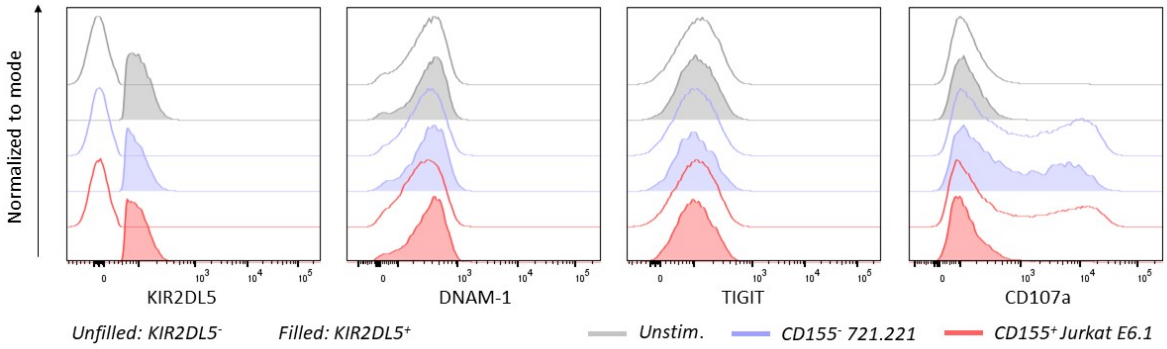

Figure 9: Expression patterns of CD155-binding NK cell receptors and NK cell degranulation in response to CD155$^-$ (721.221) and CD155$^+$ (Jurkat E6.1) target cells. NK cell receptor (KIR2DL5, DNAM-1, TIGIT) and CD107a expressions are depicted as histograms showing the fluorescence intensities on NK cells. Expression patterns are shown for KIR2DL5$^-$ NK cells (unfilled) and KIR2DL5$^+$ NK cells (filled) that were unstimulated (gray), co-incubated with CD155$^-$ 721.221 cells (blue) or CD155$^+$ Jurkat E6.1 cells (red).

A specific interaction between KIR2DL5 and CD155 was reported by using transfected cell lines and surface plasmon resonance (SPR) [39,40]. However, the affinity of KIR2DL5-binding to CD155 remains currently unknown. In the initial screening identifying the novel interaction, the binding of CD155 to DNAM-1 and TIGIT was stronger compared to interactions with KIR2DL5 [39]. Even though the binding affinity of DNAM-1 to CD155 seems to be higher, we observed that inhibitory signals mediated through KIR2DL5 binding to CD155 were overcoming activating signals, which is in line with the dominant inhibition of NK cells mediated by HLA specific inhibitory KIRs [31]. While KIR2DL5 binds to a non-classical ligand, the functional features of the receptor seem to be similar to those of other ITIM-containing inhibitory KIRs.

KIR2DL5 is expressed on up to 10% of peripheral blood NK cells in healthy individuals encoding for the expressed *KIR2DL5A*001* gene [57], while other receptors recognizing CD155 exhibit a broader expression pattern [160,161]. To study KIR2DL5$^+$ NK cell function, we either used KIR2DL5 specific antibodies to distinguish between KIR2DL5$^-$ and KIR2DL5$^+$ NK cells via flow cytometry, or generated NK cell clones either positive or negative for KIR2DL5 on the cell surface. In viral inhibition experiments we used NK cell clones to ensure that the NK cells employed were either 100% positive or negative for KIR2DL5 expression (Figure 10). This enabled a direct comparison of KIR2DL5$^+$ and KIR2DL5$^-$ NK cell function, without having mixed effects. Using NK cell clones furthermore enabled us to phenotypically characterize the NK cells for co-expression of the other known CD155-interacting NK cell receptors TIGIT, DNAM-1 and CD96. While the NK cell clones differed significantly in KIR2DL5 expression (p value = 0.002), no significant differences between the two groups for the other markers were observed (Figure 10).

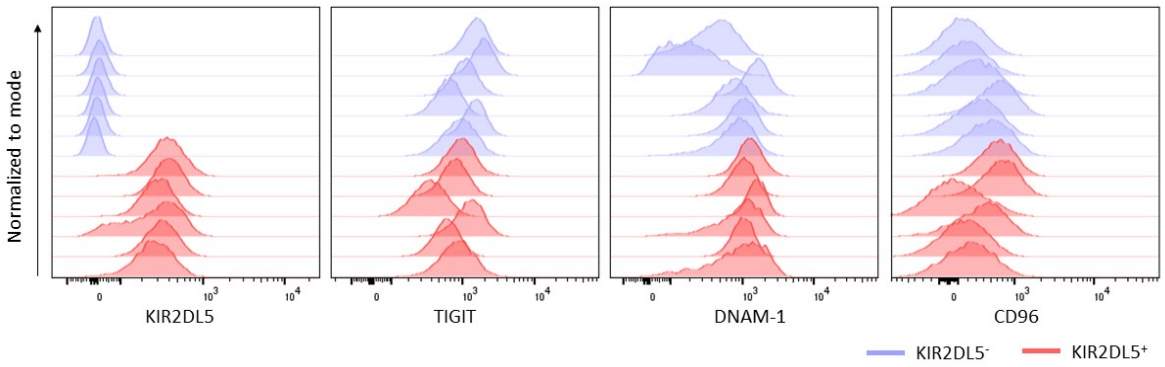

Figure 10: Phenotyping of KIR2DL5$^-$ and KIR2DL$^+$ NK cell clones. KIR2DL5$^-$ NK cell clones (blue) and KIR2DL5$^+$ NK cell clones (red) were stained for KIR2DL5, TIGIT, DNAM-1 and CD96 expression. Receptor expression levels are depicted as histograms showing the fluorescence intensities.

The fact that only a minority of *KIR2DL5* genes are transcribed and even fewer are expressed on the cell surface (mainly KIR2DL5A*001) [57] may explain why a genetically polymorphic receptor interacts with a conserved molecule such as CD155. However, as discussed above, KIR2DL5 potentially also binds to more polymorphic ligands, such as HLA molecules, under specific conditions, e.g. a certain peptide repertoire.

It has been reported that KIR2DL5 expression is not correlated with the expression of other KIRs [56,63]. Furthermore, the existence of NK cell subsets expressing KIR2DL5 but no other inhibitory receptors like NKG2A or KIRs has been described [57]. This raises the question whether NK cells can be licensed via KIR2DL5 and more specifically through the interaction of KIR2DL5 with CD155. Mechanisms of NK cell education via interactions of NK cell receptors with non-classical and non-HLA ligands have been indicated [42]. The existence of NK cells only expressing KIR2DL5 as an inhibitory receptor indicates a capacity of KIR2DL5 to license NK cells. This assumption is further strengthened by the observation of increased numbers of KIR2DL5$^+$ NK cells and a retained cytotoxic capacity of resting NK cells in a patient with TAP-deficiency [57,152]. TAP-deficiency results in loss of HLA class I surface expression [153]. Therefore, an HLA class I-dependent education is not possible and the retained cytotoxic capacity together with increased KIR2DL5 levels indicate a KIR2DL5 and non-HLA class I-mediated education of those NK cells, potentially by interactions of KIR2DL5 with CD155. However, further functional analyses are required to unravel a potential impact of KIR2DL5 in combination with or without CD155 on NK cell education.

3.2 KIR2DL5 in HIV-1 infection

Due to the fact that no functional ligands were known for a long time, little is known about the impact of KIR2DL5 on human health, the course of infections and the development of autoimmune diseases. In our study, we observed that the HIV-1-mediated downregulation of CD155 on infected cells results in an increased antiviral activity of KIR2DL5$^+$ NK cells, showing for the first time functional consequences of KIR2DL5-CD155 interactions in an *in vitro* infection model.

So far, only a few genetic studies have suggested associations between individuals encoding for *KIR2DL5* and the outcome of infectious diseases. While in some studies the existence of a *KIR2DL5* gene was suggested to be beneficial for the outcome of infections, others observed an increased susceptibility to viral infections or a reduced response to treatments (table 1). Recent studies showed an association between KIR2DL5 and a reduced mother-to-child transmission of HIV-1 in infants born by HIV-1-infected mothers [109] and in HIV-1-exposed but uninfected infants [110], supporting my hypothesis of the importance of KIR2DL5 antiviral activity. In contrast, another genetic study describes a higher susceptibility of *KIR2DL5*-positive individuals to viral infections such as HIV-1 [111]. Furthermore, there is evidence that *KIR2DL5*-positive individuals have a higher potential to spontaneously clear HBV infection [162,163]. Additional studies described an increased susceptibility to HCV [112] and non-response to antiviral treatment [164]. Interestingly, KIR2DL5 was associated with decreased susceptibility to HCMV infection [165]. This is in particular noteworthy as HCMV is able to downregulate CD155 [83], potentially representing an additional virus that is aiming to escape DNAM-1-mediated NK cell recognition and thereby becomes vulnerable to recognition by KIR2DL5$^+$ NK cells. Further studies describe an increased susceptibility as well as a beneficial effect on the course of malaria infection [166,167], and an association with severe influenza A (H1N1) infections [168]. Nevertheless, as there was no discrimination between *KIR2DL5A* and *KIR2DL5B* alleles in most studies, the relevance of the results remains elusive. In future genetic association studies, a discrimination between the different *KIR2DL5* gens should be considered with focus on the expressed *KIR2DL5A*001*. Furthermore, many of the before mentioned studies analyzed populations of different geographical regions. As KIR2DL5 allele frequencies vary between different populations [57], the results might not be applicable for all human populations.

Table 1: Genetic associations of *KIR2DL5* and infectious diseases:

Infection	Observation	Reference
HIV-1	Decreased susceptibility	[110]
	Decreased susceptibility	[109]
	Increased susceptibility	[111]
HBV	Viral clearance	[162]
	Viral clearance	[163]
HCV	Increased susceptibility	[112]
	Decreased response to antiviral treatment	[164]
CMV	Decreased susceptibility	[165]
Malaria	Increased susceptibility	[166]
Malaria	Beneficial for course of infection	[167]
Influenza A	Associated with severe pandemic influenza A (H1N1) 2009 infections	[168]

In our studies, we focused on the impact of KIR2DL5 on HIV-1 infection by investigating the antiviral activity of KIR2DL5$^+$ NK cells. Some tumors and viruses downmodulate HLA class I on the cell surface, mainly to evade recognition by cytotoxic T cells, and thereby become targets for NK cells. HIV-1 evolved several mechanisms to modulate the expression of surface proteins that act as activating ligands for immune cell receptors to impair immune recognition of infected cells, including the downregulation of HLA class I molecules and stress induced ligands such as the NKG2D ligands MICA, ULBP1 and ULBP2 [169–173]. HIV-1 encodes for the two accessory proteins Nef and Vpu, which primarily function to alter the presence and location of cell surface proteins to increase viral fitness and to escape immune recognition. We found that HIV-1 strains downmodulate CD155 on the surface of infected cells, which serves as a ligand for KIR2DL5 [41]. Other studies also suggested a modulation of CD155 by HIV-1. However, the impact of HIV-1 on CD155 levels was discussed controversially, as some studies described a downmodulation [82,150], while others showed increased levels of surface CD155 [80] or no changes at all [151]. As most experiments were performed by using cell lines and pseudotyped viruses, we decided to analyze the capability of lab-adapted and primary full-length HIV-1 strains to regulate CD155 surface levels on primary human CD4$^+$ T cells. It might seem counterintuitive that virus-induced downregulation of CD155 renders cells more vulnerable to killing by KIR2DL5$^+$ NK cells. However, downregulation of CD155 by HIV-1 has probably evolved to avoid DNAM-1-mediated NK recognition, at the same time rendering HIV-1-infected cells

more vulnerable to KIR2DL5$^+$ NK cells. In additional experiments using Nef-deficient viruses, we observed that the viral protein Nef is involved in the downregulation of CD155 [41]. Nef is a membrane-associated protein expressed early in infection. Nef induces the downregulation of CD4 by recruiting the clathrin adaptor protein AP-2 leading to endocytosis of CD4 and ultimately lysosomal degradation (Figure 11). In addition, Nef downmodulates HLA class I molecules and especially HLA-A and HLA-B molecules from the cell surface to inhibit adaptive immune responses, preventing recognition and killing by HIV-specific CD8$^+$ T cells [135] (Figure 11). Two mechanisms of Nef-mediated downregulation of HLA class I have been suggested. Nef might either mediate complex formation with AP-1 in the golgi aparatus, resulting in endocytosis and lysosomal degradation [174] and/or actively remove HLA class I molecules from the plasma membrane [135] (Figure 11). The exact mechanisms involved in the downmodulation of CD155 are unknown. However, there is evidence that Nef uses the same motifs to downregulate CD155 as for the downregulation of HLA class I molecules [82].

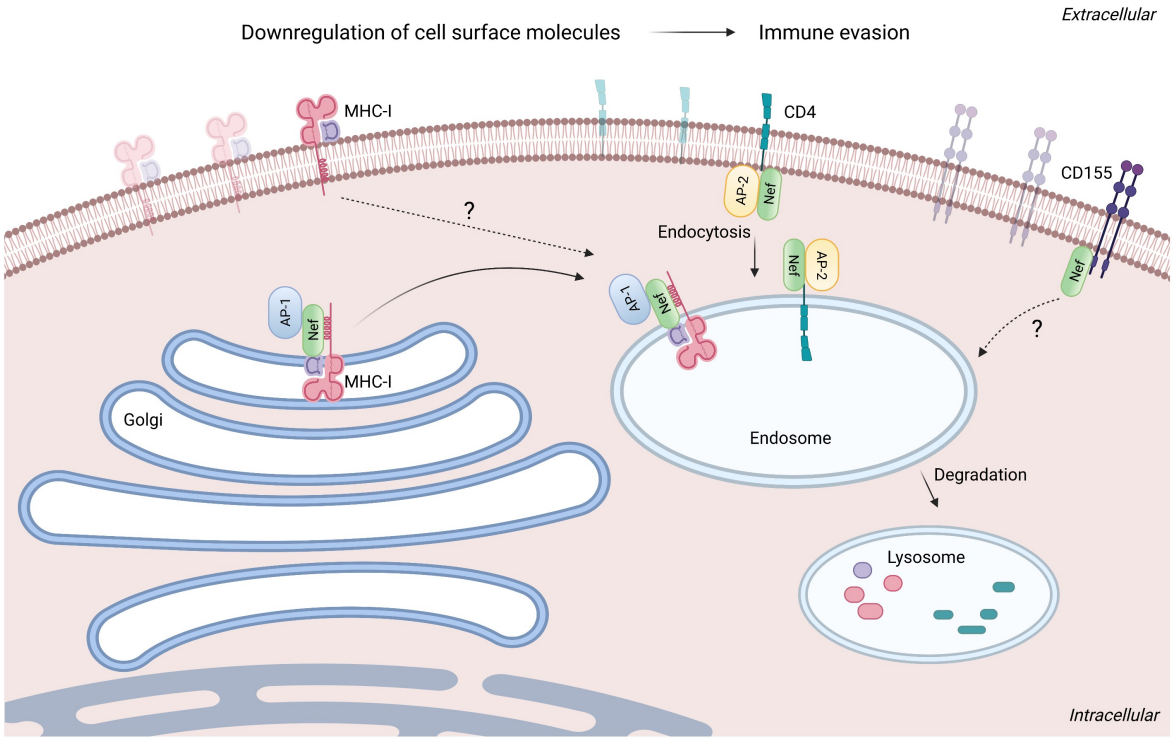

Figure 11: The viral protein Nef downregulates host-cell surface molecules to facilitate immune evasion. Nef induces downregulation of MHC class I molecules by binding to AP-1 and MHC-I, leading to endocytosis and lysosomal degradation. A potential additional mechanism is the downregulation directly from the cell surface by internalization and retention at the trans-golgi network. For the downregulation of CD4, Nef recruits AP-2 linking CD4 to clatherin-mediated endocytosis resulting in lysosmal degradation. The exact mechanism involved in Nef-mediated downregulation of CD155 is unknown.

In addition to Nef, it has been reported that the viral protein Vpu might also be involved in downregulation of CD155 [82,150], while others described no involvement of Vpu in CD155 modulation or exclusively for individual patient isolates [151,175]. Beside the effect of Nef on CD155 levels, we therefore also investigate Vpu-mediated downregulation of CD155 on infected cells, using Vpu-deficient and Nef/Vpu double knock out (KO) viruses of the primary patient isolate CH198 (Figure 12). Our data suggested a minor effect of Vpu on the cell surface expression of CD155, as CD155 levels were slightly (but not significantly) increased on cells infected with the Vpu-deficient virus compared to cells infected with the wild type virus. By using a Nef/Vpu double KO virus, this effect was more pronounced, indicating that Vpu together with Nef might be involved in the modulation of CD155 expression. However, substantially more experiments and data would be required to investigate possible functional consequences for NK cells.

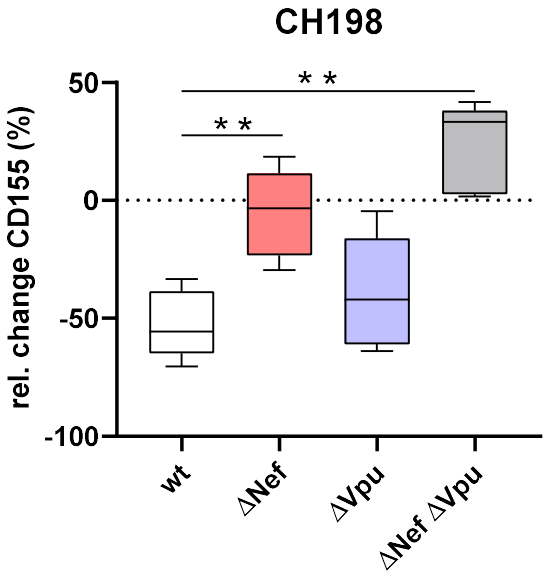

Figure 12: Impact of HIV-1 Nef and Vpu on the downregulation of CD155 on infected CD4⁺ T cells. HIV-1 CH198 wild type (wt) was compared to ΔNef, ΔVpu and ΔNef/ΔVpu mutant viruses and analyzed for their ability to downmodulate CD155. The relative change of CD155 expression is shown for wt (white), ΔNef (red), ΔVpu (blue) and ΔNef/ΔVpu (grey) mutant viruses. Mann-Whitney test was used to calculate differences between the downregulation of CD155 by CH198 wt, ΔNef, ΔVpu and ΔNef/ΔVpu mutant viruses.

The different viral strains analized in our study showed variations in the degree of CD155 downregulation. It is known that different HIV-1 strains exhibit varying potentials to modulate cell surface molecules on infected cells, including HLA class I and HLA-E [176–178]. A reason might be that viruses used within the study were isolated at different time points after infection, and therefore evolved under different immune pressures mediated by NK

cells or $CD8^+$ T cells. In our panel we included a chronic patient isolate (CH293), which showed the lowest ability to downmodulate CD155, indicating no advantage to maintain this function at a later stage of infection, when the immune pressure is impaired. While the Vpu-deficient HIV-1 strain was not sufficient to completely abrogate CD155 downregulation (Figure 12), the stong effect observed when infecting with a Nef/Vpu double KO virus might suggest a synergic effect of Nef and Vpu on the downregulatory capacity of HIV-1. This is in line with a rising evidence, that the viral proteins Nef and Vpu partially function synergistically in an effort to escape immune recognition. In addition to a potential involvment of Nef and Vpu in CD155 downregulation [41,82], both proteins have also been shown to be involved in the downregulation of CD4 and the escape from ADCC [179,180]. Additionally, the involvemnt of the early viral protein Nef together with the late viral protein Vpu might be a mechanism to ensure continuous downregulation. However, our data do not suggest a clear role of Vpu, and we therefore cannot draw any conclusions on a possible involvement of Vpu.

To further analyze the Nef-mediated downregulation of CD155, we investigated the antiviral activity of $KIR2DL5^+$ NK cells in an *in vitro* model. By co-culturing $CD4^+$ T cells infected with wild type or Nef-deficient HIV-1 strains together with autologous $KIR2DL5^+$ or $KIR2DL5^-$ NK cell clones, we observed a significantly impaired inhibition of viral replication by $KIR2DL5^+$ NK cells in the absence of Nef compared to wild type viruses encoding for a functional Nef that downregulates CD155. $KIR2DL5^-$ NK cell populations also showed a slightly but not significantly reduced inhibition of Nef-deficient viruses. It has been described that Nef is important for viral replication and that Nef-deficent HIV-1 strains might therefore have a reduced replication capacity [176,181–184]. However, comparing the inhibition of viral replication of the Nef-deficient CH198 virus between the different NK cell populations, $KIR2DL5^+$ NK cells showed a significantly lower antiviral activity against Nef-deficient viruses compared to $KIR2DL5^-$ NK cells, indicating a KIR2DL5-specific effect independent from a possible reduced viral replication capacity of Nef-deficient viruses.

Taking this together, we conclude that HIV-1 wild type infection leads to a Nef-dependent downregulation of CD155 on infected $CD4^+$ T cells, resulting in loss of inhibition and subsequent antiviral activity of $KIR2DL5^+$ NK cells (Figure 13, left). Nef-deficient HIV-1 is not capable of downregulating CD155 leading to inhibition of $KIR2DL5^+$ NK cells and a reduced antiviral activity (Figure 13, right).

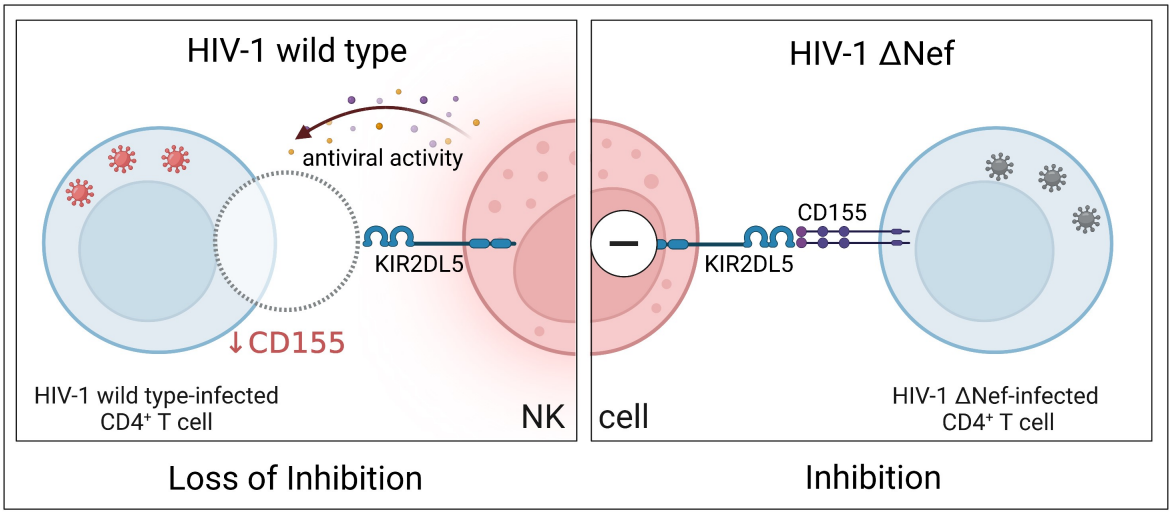

Figure 13: HIV-1 Nef-mediated downregulation of CD155 results in antiviral activity of KIR2DL5$^+$ NK cells. CD155 inhibits the antiviral activity of KIR2DL5$^+$ NK cells and is downmodulated by HIV-1 Nef protein. Downregulation of CD155 by HIV-1 wild-type results in loss of inhibition and enhanced antiviral activity of KIR2DL5$^+$ NK cells (left). Nef-deficient HIV-1 (ΔNef) does not downregulate CD155, leading to inhibition and reduced antiviral activity of KIR2DL5$^+$ NK cells (right).

In addition to Nef and a possible involvement of Vpu, Vassena et al. suggested the viral protein Vpr to be also involved in modulating CD155 surface levels [80]. Unexpectedly, this study described a Vpr-mediated upregulation of CD155 expression on HIV-1-infected cells via an activation of the ATR-mediated DDR pathway [80]. Increasing evidence indicate that CD155 can function as a stress-induced molecule, upregulated upon induction of the DDR in several settings [74,185]. Vpr induces a G_2 cell-cycle arrest and the activation of the DDR, which is a highly conserved process and thought to be important for virus production [186,187]. One could speculate that the upregulation of CD155 is a consequence of the Vpr-mediated activation of the DDR, which is counteracted by Nef (and potentially Vpu) to avoid DNAM-1-mediated immune recognition [80].

While we focused on the consequences of CD155 downregulation on HIV-1-infected cells for KIR2DL5$^+$ NK cells, another important CD155 binding partner is the inhibitory NK cell receptor TIGIT. TIGIT is up-regulated by NK cells in response to HIV infection [188,189]. However, Vendrame et al. observed that TIGIT does not directly participate in the anti-HIV response, as blocking of TIGIT had no effect on the anti-HIV response of NK cells [188]. To control for TIGIT expression on NK cells during our experiments assessing the antiviral activity of KIR2DL5$^+$ NK cells, we used NK cell clones that were separated into KIR2DL5$^+$ and KIR2DL5$^-$ NK cells, while TIGIT was expressed on both NK cell populations to the same extend (Figure 10), enabling the investigation of a KIR2DL5-specific effect.

Of note, HIV-1 does not only infect CD4$^+$ T cells but also macrophages [190,191], which do constitutively express CD155 [192]. Also on macrophages CD155 levels were decreased

during HIV-1 infection (data not shown, unpublished), providing a potential additional site of action for KIR2DL5$^+$ NK cells that requires further investigation.

Taking together, we showed that KIR2DL5$^+$ NK cells can sense changes of CD155 levels on target cells, leading to increased antiviral activity in response do HIV-1 Nef-mediated downregulation of CD155. These findings demonstrate the importance of KIR2DL5-CD155 interactions for the anti-HIV-1 effector function of NK cells from KIR2DL5-positive individuals and furthermore indicate a relevance of the interaction for other viral infections and in cancer.

3.3 Impact of KIR3DS1-HLA-F interactions on HCV infection

NK cells play a key role in the early control of HCV infection [143]. Genetic association studies repeatedly showed an association between individuals encoding for the activating NK cell receptor KIR3DS1 and the disease outcome including spontaneous clearance of HCV infection and a reduced risk to develop hepatocellular carcinoma [91,96]. In our study, we investigated the underlying mechanisms involving the impact of KIR3DS1-HLA-F interactions on the KIR3DS1-mediated immune response of NK cells during HCV infection. We observed that the KIR3DS1 ligand HLA-F is upregulated on HCV-infected cells by using cell culture systems, humanized mice and primary liver tissue from HCV-infected patients [94]. Finally, we showed that KIR3DS1-HLA-F interactions contribute to the NK cell-mediated control of HCV [94].

In contrast to KIR2DL5 and most other KIRs, KIR3DS1 is genetically rather monomorphic [84]. Furthermore, HLA-F has a low polymorphism in comparison to other HLA class I molecules [99], implicating a conserved interaction between the two molecules. Both, KIR3DS1 as a monomorphic KIR and KIR2DL5 as more polymorphic KIR, which however is only expressed by very few alleles (mainly KIR2DL5A*001), indicate unique features in comparison to other highly polymorphic KIRs. KIR3DS1 binds to OCs of the non-classical HLA class I molecule HLA-F [51]. In contrast to other KIRs, KIR3DS1 as well as KIR2DL5 do interact with conserved ligands that are upregulated upon activation and cellular stress [74,100,185]. However, while CD155 is constantly expressed at low levels in many cells and tissues [193,194], the expression of HLA-F is tightly regulated and HLA-F is not present on the cell surface under normal conditions [100]. The fact that KIR3DS1 binds to OCs of HLA-F strengthens the assumption that the interaction between KIR3DS1 and HLA-F OCs is a conserved mechanism. Even though HLA-F OCs are stable at the cell surface, they do not present peptides, showing that the interaction is independent from any specific peptides or peptide variability. This makes viruses unable to abrogate the interaction by altering the peptide repertoire. The observed upregulation of HLA-F on HCV-infected cells *in vitro* and

in vivo is in line with other studies showing an upregulation of HLA-F in consequence of several other persistent viral infections including HIV-1, HAdV and BKPyV and a subsequent antiviral activity of KIR3DS1$^+$ NK cells in response to infected cells [51,93–95]. Interestingly, all four viruses have the ability to downmodulate the expression of surface molecules to evade immune recognition and to establish persistent viral infections in humans, including the downregulation of NKG2D ligands [195]. HCV uses the viral proteins NS2 and NS5B to diminish MICA and MICB expression [196] and HIV-1 utilizes Nef to target MICA, ULBP1 and ULBP2 expression [197]. Furthermore, HIV-1 and HAdV are capable of downregulating HLA class I molecules from the surface of infected cells, leading to evasion of T and NK cell recognition [135,178,198,199]. However, HLA-F expression levels seem not to be modulated in order to avoid KIR3DS1-dependant NK cell recognition. Only HIV-1 might have evolved some degree of interference with HLA-F expression, as HIV slightly decreased KIR3DS1 ligand expression during late stage of infection, suggesting a potential evasion mechanism probably involving HIV-1 accessory proteins, similar to the mechanisms observed for the downregulation of HLA class I and NKG2D ligands like MIC-A [51]. In conclusion, KIR3DS1-HLA-F recognition seems not to represent a strong immune pressure, which would initiate the development of specific evasion mechanisms. A reason for that might be the lower frequencies of *KIR3DS1* carriers, as *KIR3DS1* is encoded by around 40% (ranging from 0.7% to 80%) of individuals in different populations [200]. The lower frequencies of *KIR3DS1* compared to other more abundantly expressed KIRs might constitute a lower immune pressure for viruses, resulting in less immune evasion strategies to abrogate KIR3DS1-mediated NK cell activation. Additional *in vitro* and *in vivo* investigations are required to further evaluate the impact of KIR3DS1-HLA-F interaction on the course of other viral infections and to clarify whether viruses interfere with HLA-F expression. Nevertheless, KIR3DS1 seems to be an important factor for an improved antiviral response, as association studies describe *KIR3DS1* alone and in combination with *HLA-Bw4* to be beneficial in several viral infections (Table 2). HLA-Bw4 serves as a ligand for the inhibitory KIR3DL1, which is co-expressed by more than 90% of KIR3DS1$^+$ individuals [50,85]. The described protective effect of *KIR3DS1* in combination with *HLA-Bw4* might therefore be explained by KIR3DL1-mediated licensing of KIR3DS1$^+$ NK cells that subsequently become activated in response to HLA-F expression [201,202].

Table 2: Genetic associations of *KIR3DS1* with viral infections:

Infection	KIR (and HLA)	Observation	Reference
HCV	KIR3DS1	Viral clearance Sustained virological response	[91]
	KIR3DS1/HLA-Bw4	Viral clearance	[114]
HIV	KIR3DS1/HLA-Bw4	Delayed progression to AIDS	[89]
	KIR3DL1/S1/HLA-Bw4	Slower disease progression	[90]
	KIR3DL1/S1/HLA-Bw4	Viral inhibition	[203]
	KIR3DS1/KIR3DS1	Viral resistance	[113]
HBV	KIR3DS1	Viral clearance	[162]
	KIR3DS1	Viral resistance Spontaneous remission	[204]
HPV	KIR3DS1	Viral resistance	[205]
BKPyV	KIR3DS1	Viral resistance	[92]
EBV	KIR3DS1	Viral susceptibility	[206]
	KIR3DS1	Viral susceptibility	[207]
Dengue virus	KIR3DS1/KIR3DS1	Viral resistance	[208]
KSHV	KIR3DS1/HLA-Bw4	Viral clearance	[209]

In our study we observed that upon upregulation of HLA-F on HCV-infected hepatocytes KIR3DS1$^+$ NK cells exhibited a higher capacity to inhibit viral replication *in vitro*, potentially leading to an improved clearance of HCV infection [94] (Figure 14, left). These findings provide a functional mechanism behind the before described genetic associations between a better outcome of HCV infection and individuals encoding for *KIR3DS1*. In contrast, KIR3DS1$^-$ NK cells had a lower ability to inhibit viral replication, indicating that individuals lacking KIR3DS1 have a normal course of disease (Figure 14, right).

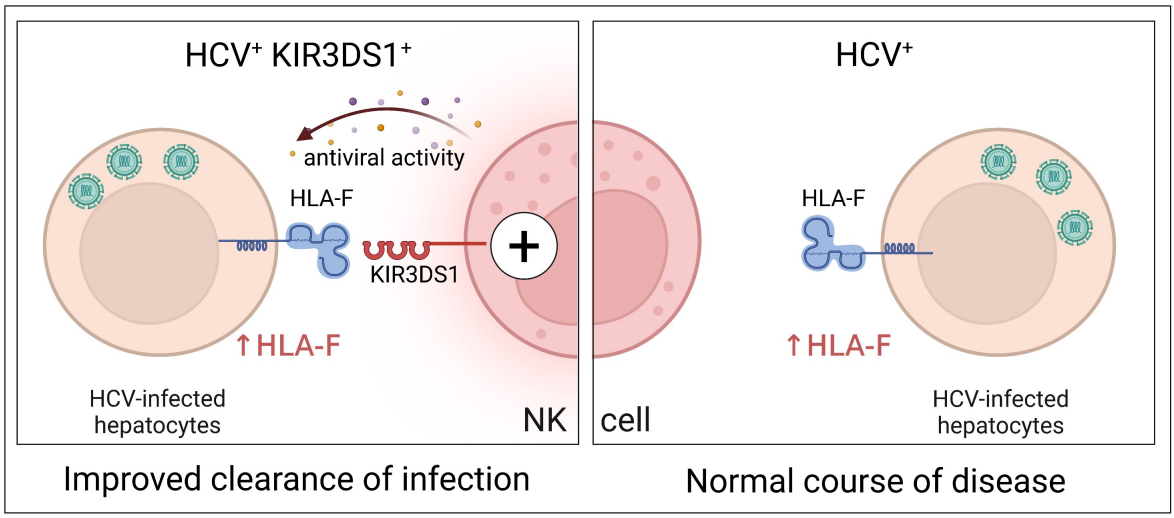

Figure 14: Interactions between KIR3DS1 and HLA-F contribute to the control of HCV infection by KIR3DS1⁺ NK cells. HLA-F surface expression is upregulated on HCV-infected hepatocytes. The upregulation can be recognized by KIR3DS1⁺ NK cells leading to antiviral activity and an improved clearance of HCV infection by individuals encoding for *KIR3DS1* (left). In contrast, patients lacking KIR3DS1 do not sense HLA-F upregulation and have a normal course of infection (right).

HLA-F expression is tightly regulated and the exact mechanisms behind it are not fully understood. Similar to the expression of CD155, there is increasing evidence that HLA-F surface expression is induced through activation and cellular stress [99], potentially adding another molecule to the family of stress-induced self-ligands such as MIC and ULBP [210,211]. Interestingly, both, expression of HLA-F and CD155 was found to be upregulated in a number of cancers and are described as poor prognostic markers [76,212,213], making them an interesting target for studying the influence of these molecules on NK cell activity in tumors.

We observed a clear upregulation of HLA-F on HCV-infected cells in all three infection models (cell culture, humanized mice and primary liver tissue) while HLA-F expression on uninfected bystander cells and liver biopsy sections from HCV^{neg} controls was very low or completely absent [94]. However, the mechanism leading to HLA-F surface expression during viral infections are not well known. HLA class I gene expression is among other mechanisms regulated trough the IFN-stimulated response element (ISRE) that serves as a regulatory element in the promoter of classical and non-classical HLA class I genes and is induced through IFNγ [214,215]. An additional mechanism that is induced through IFNγ and has been discussed to be able to induce HLA-F expression is the transcriptionally regulation via CIITA [215]. Furthermore, HLA-F gene expression can be induced by exogenous IFNγ [216]. IFNγ is a pro-inflammatory cytokine with important antiviral activity [217]. However, HCV infection is associated with IFN class I and III production by infected cells and less with high levels of IFNγ [143]. Nevertheless, IFNγ is produced by NK cells

during HCV infection [218–220]. Therefore, the involvement of ISRE and CIITA in HLA-F upregulation during HCV infection would be possible. Furthermore, it has been shown that classical HLA class I molecules are upregulated upon HCV infection that might suggest a related mechanism [221].

In comparison to classical HLA class I molecules, which are predominantly expressed at the cell surface as peptide loaded heavy chains associated with β_2m, HLA-F is predominantly expressed as OCs [103,104]. While surface expression of classical HLA class I complexes is TAP dependent, HLA-F OC expression is TAP independent [222,223]. Furthermore, it has been suggested that the cytoplasmic tail of HLA-F has a unique structure and is essential for the export from the endoplasmic reticulum (ER) [211]. This might indicate that to date unknown mechanisms target specific structures of HLA-F upon cellular stress or activation leading to HLA-F surface expression, while the involvement of TAP dependent mechanisms is unlikely, especially for OCs. An additional theory that could explain the limited surface expression of HLA-F under normal conditions is the retention of HLA-F in the ER [216,224]. ER stress might initiate the transport of trapped HLA-F to the cell surface during specific conditions such as viral infections. Viruses are known to induce different ER stress [225]. As an example, HBV triggers different pathways of the unfolded protein response compared to HCV [226,227] and HLA-F surface expression is not upregulated during HBV infection (unpublished data from our group) but upon HCV infection [94], potentially representing another mechanism that could be involved in the upregulation of HLA-F surface levels during HCV infection. There is evidence that HLA-F expression might partially be regulated through the transcription factor NFκB [214] and that induction of NFκB during viral infection can lead to an upregulation of HLA-F [228]. Indeed, an induction of NFκB activation during HCV infection has been shown [229], providing an additional potential mechanism involved in HLA-F upregulation during HCV infection. In summary, surface expression on infected cells is probably induced by combined intrinsic and extrinsic factors.

The mechanisms involved in the modulation of surface expression of HLA-F during HCV infection do essentially differ from the direct regulation of CD155 expression employed by HIV-1. HCV does probably not actively interfere with HLA-F surface expression and the observed upregulation during infection is driven by host intrinsic factors due to infection and following activation of the infected cell, leading to increased susceptibility to recognition by KIR3DS1$^+$ NK cells. In contrast, the modulation of CD155 surface expression during HIV-1 infection is a result of an active mechanism evolved by HIV-1 potentially in an effort to evade immune recognition by DNAM-1$^+$ NK cells. DNAM-1 is expressed on most human NK cells [160], while KIR3DS1 expression is limited due to lower carrier frequencies and a limited

amount of NK cells expressing the receptor [84,230,231]. One could speculate that this leads to different immune pressures regarding the maintenance of viral fitness and may explain why CD155 expression but not HLA-F expression is targeted by the respective virus. Taking this together, more studies are required to unravel the factors that are involved in HLA-F upregulation during HCV and other viral infections.

3.4 Interactions between KIRs and non-classical ligands as potential targets for immunotherapeutic approaches

Based on the discovery of novel receptor-ligand interactions and their functional impact on NK cell activity, new targets for antiviral therapies can be identified and the respective receptor or ligand can be utilized for the development of novel therapy approaches, such as reagents blocking inhibitory KIRs or facilitating specific activation [94,232,233]. In this dissertation, we provide new insights into the regulation of KIR2DL5$^+$ and KIR3DS1$^+$ NK cell function by the interaction with non-classical KIR ligands and how these interactions can contribute to the control of HIV-1 and HCV infections. The newly described and here validated interactions between KIR2DL5 and CD155 reveal a new target for checkpoint inhibitors in multiple settings of immunotherapeutic approaches. Other inhibitory immune checkpoint receptors are already largely under investigation as potential targets to overcome NK cell and T cell inhibition during viral infections and cancer [234–236]. The inhibitory NK cell receptor TIGIT, which is also binding to CD155, has been proposed as a potential target for anti-HIV therapy [235]. It was shown that the blockade of TIGIT might be beneficial and increases antiviral effector functions [235]. During the course of infection, inhibitory receptors are often upregulated and effector cell functions become impaired [237,238]. Indeed, during HIV-1 infection an upregulation of TIGIT on infected cells has been reported [188,189]. As TIGIT also binds to CD112 [69], beside CD155 that is downregulated during HIV-1, the blockage of TIGIT might serve as a promising therapeutic approach. The interaction of KIR2DL5 with CD155 provides another promising target for therapeutic approaches. However, we hypothesize that the expression of KIR2DL5 is beneficial for patients with HIV-1 infection as the HIV-1-mediated downregulation of CD155 leads to a loss of inhibition and a missing self-recognition of HIV-1-infected cells by KIR2DL5$^+$ NK cells. In future studies it has to be investigated whether individuals encoding for the expressed *KIR2DL5A*001* gene have a higher potential to control HIV-1 infection leading to a slower disease progression. Therefore, large HIV cohort studies are required that distinguish specifically between expressed und unexpressed *KIR2DL5* alleles to gather further insights into the antiviral potential of KIR2DL5$^+$ NK cells. While CD155 is downregulated during HIV-1 infection, it is overexpressed in tumors [76]. With this, cancer

represents a second very important disease setting in which KIR2DL5-CD155 interactions might play an important role. Several clinical trials already investigate TIGIT blockade as immunotherapeutic approach in cancer [236]. In future studies, the consequences of elevated CD155 levels in tumors for KIR2DL5$^+$ NK cell function should also be considered, as this might deliver another potential target for immunotherapeutic strategies, at least for patients expressing KIR2DL5.

In contrast to KIR2DL5, KIR3DS1 is an activating receptor. Therefore, the KIR3DS1-HLA-F axis represents a promising target for triggering specific activating signals. Several studies have shown that the adoptive transfer of antiviral effector cells can be used to facilitate the control of viral infections when other treatments fail or are not available [239–241]. Additionally, engineered chimeric antigen receptor (CAR) NK cells get rising attention, as they show some benefits compared to CAR-T cells, including low toxicity and easier manufacturing [242,243]. The adoptive transfer of KIR3DS1$^+$ NK cells or the use of CAR-NK cells might therefore be promising approaches for the treatment of severe viral infections or cancer. However, the use of adoptive KIR3DS1$^+$ NK cells needs to be further investigated, as unspecific side effects due to HLA-F expression in other tissues at this point cannot be excluded and other immunoregulatory functions of KIR3DS1 have to be investigated. An additional promising approach for the treatment of viral infections, which come with an upregulation of HLA-F, is the use of KIR-Fc constructs. NK cells are able to recognize the chimeric constructs bound to HLA-F via CD16/FcγRIII that is expressed on NK cells. As KIR3DS1 is expressed by approximately 40% of humans [200], the use of KIR3DS1-Fc would enable CD16-mediated effector function against HLA-F expressing target cells also for patients genetically lacking *KIR3DS1*. Indeed, our *in vitro* assessment of NK cell-mediated inhibition of HCV replication using KIR3DS1-Fc constructs resulted in an increased viral inhibition by *KIR3DS1*-negative donors [94]. Taking this together, the mentioned interactions might represent promising targets for new therapeutic approaches that require further careful investigation.

4 Conclusion

This thesis investigated the impact of novel interactions between KIRs and non-classical ligands on the antiviral activity of KIR-positive NK cell subsets. I demonstrated that CD155 serves as a functional binding partner of KIR2DL5 and that their interaction leads to decreased antiviral activity of KIR2DL5$^+$ NK cells. Furthermore, we showed that HIV-1 downregulates CD155 on the surface of infected CD4$^+$ T cells and that HLA-F is upregulated on the surface of hepatocytes following infection with HCV. Downregulation of the KIR2DL5 ligand CD155 during HIV-1 infection and upregulation of the KIR3DS1 ligand HLA-F resulted in increased antiviral activity of KIR2DL5$^+$ (missing-self recognition) and KIR3DS1$^+$ NK cell (induced-self recognition), respectively. With this, both interactions are important for the regulation of NK cell function and contribute to the recognition of infected cells and the induction of antiviral NK cell activity. Furthermore, the anti-HIV-1 activity of KIR2DL5$^+$ NK cells demonstrates the ability of NK cells to counteract viral immune escape mechanisms. The KIR2DL5/CD155 axis reveals a novel checkpoint inhibitor target and the interaction between KIR3DS1 and HLA-F can be utilized to trigger activation. As presented in our study, KIR-Fc constructs might allow recognition of HLA-F-expressing cells also by NK cells of individuals not encoding for KIR3DS1, resulting in increased viral inhibition. In summary, this thesis demonstrates that NK cells expressing KIRs that interact with non-classical ligands contribute to the control of HIV-1 and HCV infection *in vitro*, indicating a potential role also for other disease settings such as other viral infections and cancer and for the development of novel immunotherapeutic treatments.

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Illustrations created with BioRender.com

Eidesstattliche Versicherung

Declaration under oath

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertationsschrift selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

I hereby declare, under oath, that I have written the present dissertation on my own and have not used any resources or aids other than those acknowledged.

Hamburg, 15th of July 2022 

City and date | signature student

Appendix

Participation in publications

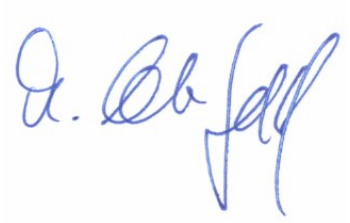
Publication #1 “HIV-1 Nef-mediated downregulation of CD155 results in viral restriction by KIR2DL5+ NK cells”, DOI: 10.1371/journal.ppat.1010572

I was involved in the design and realization of the experiments, data analysis and presentation and writing of the manuscript.

Publication #2 “Interactions Between KIR3DS1 and HLA-F Activate Natural Killer Cells to Control HCV Replication in Cell Culture”, DOI: 10.1053/j.gastro.2018.07.019

I contributed to the rebuttal of this paper by acquiring and analyzing additional experimental data.

Hamburg, 15th of July 2022



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PLOS PATHOGENS

RESEARCH ARTICLE

HIV-1 Nef-mediated downregulation of CD155 results in viral restriction by KIR2DL5$^+$ NK cells

Pia Fittje[1], Angelique Hœlzemer[1,2,3], Wilfredo F. Garcia-Beltran[1,4], Sarah Vollmers[1], Annika Niehrs[1], Kerri Hagemann[1], Glòria Martrus[1], Christian Körner[1], Frank Kirchhoff[5], Daniel Sauter[6], Marcus Altfeld[1,3]*

1 Leibniz Institute of Virology (LIV), Hamburg, Germany, **2** First Department of Internal Medicine, Division of Infectious Diseases, University Medical Center Hamburg-Eppendorf, Hamburg, Germany, **3** German Center for Infection Research (DZIF), Partner Site Hamburg-Lübeck-Borstel-Riems, Hamburg, Germany, **4** Department of Pathology, Massachusetts General Hospital/Harvard Medical School, Boston, Massachusetts, United States of America, **5** Institute of Molecular Virology, Ulm University Medical Center, Ulm, Germany, **6** Institute for Medical Virology and Epidemiology of Viral Diseases, University Hospital Tübingen, Tübingen, Germany

* marcus.altfeld@leibniz-liv.de

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Abstract

Antiviral NK cell activity is regulated through the interaction of activating and inhibitory NK cell receptors with their ligands on infected cells. HLA class I molecules serve as ligands for most killer cell immunoglobulin-like receptors (KIRs), but no HLA class I ligands for the inhibitory NK cell receptor KIR2DL5 have been identified to date. Using a NK cell receptor/ligand screening approach, we observed no strong binding of KIR2DL5 to HLA class I or class II molecules, but confirmed that KIR2DL5 binds to the poliovirus receptor (PVR, CD155). Functional studies using primary human NK cells revealed a significantly decreased degranulation of KIR2DL5$^+$ NK cells in response to CD155-expressing target cells. We subsequently investigated the role of KIR2DL5/CD155 interactions in HIV-1 infection, and showed that multiple HIV-1 strains significantly decreased CD155 expression levels on HIV-1-infected primary human CD4$^+$ T cells via a Nef-dependent mechanism. Co-culture of NK cells with HIV-1-infected CD4$^+$ T cells revealed enhanced anti-viral activity of KIR2DL5$^+$ NK cells against wild-type versus Nef-deficient viruses, indicating that HIV-1-mediated downregulation of CD155 renders infected cells more susceptible to recognition by KIR2DL5$^+$ NK cells. These data show that CD155 suppresses the antiviral activity of KIR2DL5$^+$ NK cells and is downmodulated by HIV-1 Nef protein as potential trade-off counteracting activating NK cell ligands, demonstrating the ability of NK cells to counteract immune escape mechanisms employed by HIV-1.

Author summary

HIV infection remains a global health emergency that has caused around 36 million deaths. NK cells play an important role in the control of HIV-1 infections, and are able to detect and destroy infected cells using a large array of activating and inhibitory receptors, including KIRs. Here we demonstrate that CD155 serves as a functional interaction

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Competing interests: The authors have declared that no competing interests exist.

partner for the inhibitory NK cell receptor KIR2DL5, and that KIR2DL5$^+$ NK cells are inhibited by CD155-expressing target cells. CD155 surface expression on HIV-1-infected CD4$^+$ T cells was downregulated by the HIV-1 Nef protein, resulting in increased antiviral activity of KIR2DL5$^+$ NK cells through the loss of inhibitory signals. Taken together, these studies demonstrate functional consequences of the novel interaction between KIR2DL5 and CD155 for the antiviral activity of KIR2DL5$^+$ NK cells during HIV-1 infection.

Introduction

Natural killer (NK) cells are important antiviral effector cells of the innate immune system. NK cells can recognize virus-infected cells through activating receptors and the loss of engagement of inhibitory receptors [1], enabling both tolerance against self and effective immune responses against virus-infected and tumor cells [2]. One important NK cell receptor family is the group of killer cell immunoglobulin-like receptors (KIRs), which contains several structurally related activating and inhibitory receptors. To date, all described functional ligands for KIRs constitute HLA class I molecules, including KIR2DL1 and KIR2DL3 binding to HLA-C group 2 and 1, respectively, and KIR3DL1 binding to HLA-Bw4 molecules [3–5]. While for most KIRs a functional ligand has been defined, the inhibitory KIR2DL5 was long considered an "orphan" receptor [6]. Like other KIRs, KIR2DL5 is genetically polymorphic and due to a duplication of the gene in humans encoded by two different loci on chromosome 19 designated as *KIR2DL5A* and *KIR2DL5B*. However, surface expression has been mainly detected for molecules encoded by the *KIR2DL5A* alleles [6,7]. Recently, the poliovirus receptor (PVR/CD155) has been described as a binding partner for KIR2DL5 [8,9], potentially identifying a KIR not to interact with HLA class I molecules. Given the well-established functional interactions of CD155 with the activating NK cell receptors DNAM-1 [10] and CD96 [11] and the inhibitory NK cell receptor TIGIT [12], the newly described binding of KIR2DL5 to CD155 suggests a complex regulation of NK cell activity by CD155. However, the functional consequences of KIR2DL5-CD155 interactions for primary NK cells and their antiviral activity remained unknown.

Viruses have evolved multiple strategies to evade immune cell recognition, including mechanisms to reduce the surface expression of ligands for immune cells on infected cells. These processes include the downregulation of HLA class I molecules by the HIV-1 accessory proteins Nef and Vpu to evade CD8$^+$ T cell recognition. While Nef is involved in the downregulation of HLA-A and -B, Vpu reduces the surface expression of HLA-C on HIV-1-infected cells [13–16]. However, downregulation of HLA class I molecules can result in enhanced "missing-self" recognition of infected cells by NK cells through the loss of inhibitory signals mediated by inhibitory NK cell receptors binding to HLA class I [17,18]. In addition, HIV-1 can evade NK cell recognition by decreasing surface expression-levels of ligands for activating NK cell receptors such as MICA and MICB [19,20] that serve as ligands for the C-type lectin receptor NKG2D [21]. Recent data have suggested that CD155 surface expression can also be downmodulated by HIV-1, involving the viral proteins Nef and/or Vpu, probably to evade DNAM-1-dependant NK cell recognition of infected cells [22–25]. However, modulation of CD155 surface expression by HIV-1 remains incompletely understood, as some studies also suggest an upregulation [26] or no modulation of CD155 expression [20,27]. As the recently described interaction between KIR2DL5 and CD155 indicates a more complex regulation of NK cell function through CD155, we investigated the functional consequences of HIV-1-mediated

regulation of CD155 expression for KIR2DL5$^+$ NK cells. We show that CD155 serves as an important functional ligand for the inhibitory NK cell receptor KIR2DL5 that can inhibit primary human KIR2DL5$^+$ NK cell activity. HIV-1 strains decreased CD155 expression levels on HIV-1-infected CD4$^+$ T cells through a Nef-dependent mechanism, potentially in an effort to evade DNAM-1-mediated recognition by NK cells. However, this resulted in better *in vitro* inhibition of replication of wild-type viral strains by KIR2DL5$^+$ NK cells compared to ΔNef viruses. Taken together, this study provides new functional insights into the interaction between KIR2DL5 and CD155, and the consequences for antiviral activity of KIR2DL5$^+$ NK cells during HIV-1 infection.

Results

KIR2DL5 represents an additional binding partner for CD155

KIR2DL5 is an inhibitory NK cell receptor for which functional ligands are not well defined. To assess potential binding of KIR2DL5 to various HLA class I and HLA class II molecules, we performed a bead-based screening assay. NK cell receptor Fc fusion constructs, consisting of the extracellular domain of an NK cell receptor fused to an IgG1 Fc domain, were used to stain HLA-coated beads using an array of color-coded beads coated with 97 different HLA class I and 95 HLA class II molecules [28]. The NK cell receptor KIR2DL3 that recognizes HLA-C group 1 (HLA-C1) molecules [4] was used as a positive control for the HLA class I binding assay, and the lymphocyte activation gene 3 (LAG-3) protein, which is a high affinity ligand for HLA class II molecules [29], served as a positive control for the HLA class II screen (Fig 1A). While the KIR2DL3-Fc construct did interact with HLA-C1, KIR2DL5-Fc showed no strong binding to any of the investigated HLA class I molecules. Furthermore, LAG-3 was interacting with all HLA class II molecules, while KIR2DL5 did not bind to any of the HLA class II-coated beads (Fig 1A).

Nectin and nectin-like molecules play an important regulatory role for NK cell function by interacting with several NK cell receptors including TIGIT, DNAM-1 and CD96 [30]. To investigate whether members of the KIR family do also bind to nectin(-like) molecules, binding of TIGIT-, DNAM-1-, CD96- and KIR- (KIR2DL1, KIR2DL3, KIR2DL4, KIR2DL5 and KIR3DL1) Fc constructs to CD112 (Nectin-2)- and CD155 (PVR)-coated beads was determined. Beads coated with biotin served as negative control. These experiments confirmed previously described binding of nectin(-like) molecules to TIGIT, DNAM-1 and CD96 [10–12]. TIGIT and DNAM-1 exhibited the strongest interaction with CD155 and also bound to CD112 with lower affinity. CD96 is an intermediate affinity ligand for CD155 [30], and we also observed CD96-Fc construct binding to CD155 and no binding to CD112. Most investigated KIR-Fc constructs (KIR2DL1, KIR2DL3, KIR2DL4 and KIR3DL1) did not show any interaction with biotin-, CD112- or CD155-coated beads. However, KIR2DL5-Fc constructs exhibited binding to CD155, showing a slightly lower binding signal compared to DNAM-1 and TIGIT (Figs 1B and S1). Taken together, these data demonstrate that KIR2DL5, unlike other KIRs, does not strongly interact with HLA class I molecules, but binds to CD155.

CD155 serves as a functional ligand for KIR2DL5

While binding of KIR2DL5 to CD155 has previously been shown [8,9], we were interested in investigating the functional consequences of this interaction for primary NK cells. We therefore generated a KIR2DL5-expressing reporter cell line to further validate the interaction on a cellular basis by fusing the extracellular domain of KIR2DL5 to the intracellular part of the CD3ζ chain and stably expressing the chimeric construct within Jurkat cells. KIR2DL5 receptor binding to respective ligands can be determined based on CD69-upregulation on the

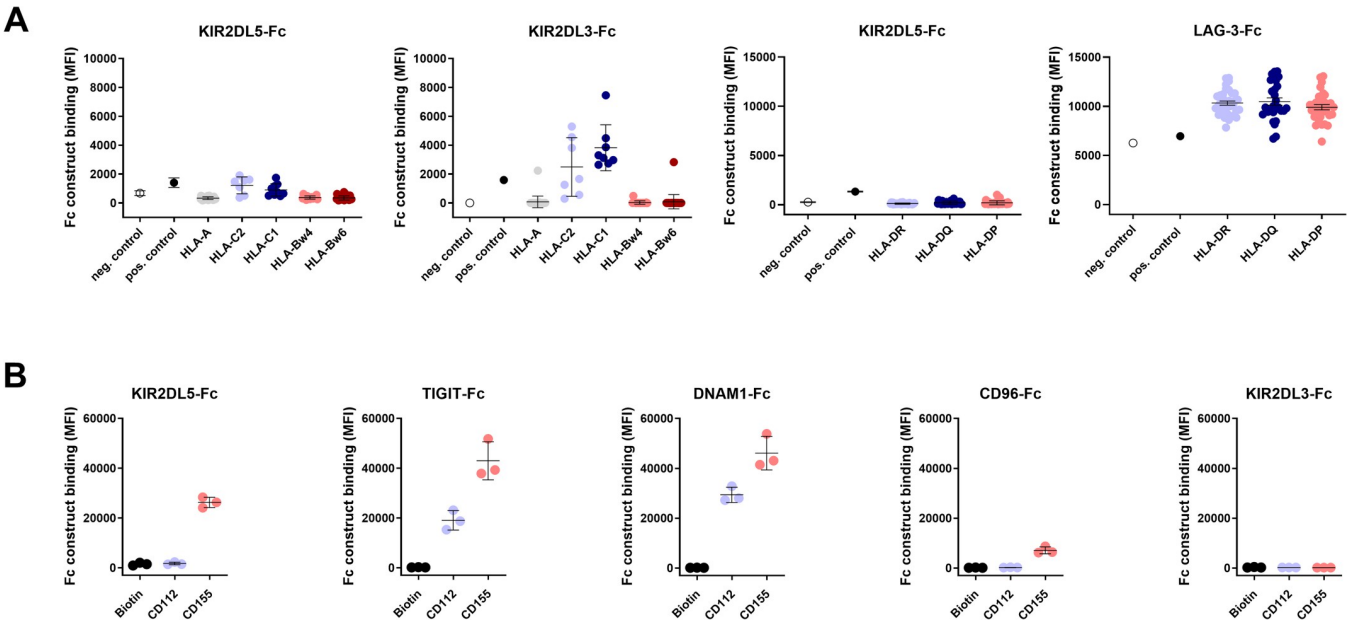

Fig 1. Binding of KIR-, TIGIT-, DNAM-1- and CD96-Fc constructs to HLA class I-, HLA class II- and nectin(-like)-coated beads. (A) KIR2DL5-Fc construct binding to HLA class I-coated and HLA class II-coated beads was measured by using a PE-labeled anti-IgG antibody and is shown as median fluorescence intensity (MFI). Each dot represents an individual HLA class I or class II allotype. Binding of KIR2DL5-Fc constructs was assessed in triplicates ($n = 3$). MFIs are shown as mean values for each HLA allotype. The 97 HLA class I allotypes as well as the 95 HLA class II allotypes were grouped according to the different subsets HLA-A, HLA-C1, HLA-C2, HLA-Bw4, HLA-Bw6 (HLA class I) and HLA-DR, HLA-DQ, HLA-DP (HLA class II). Negative control beads (white) were not coated with HLA antigen, positive control beads (black) were coated with purified human IgG. Black bars represent the mean of each HLA group and error bars show the standard deviation. (B) NK cell receptor Fc construct binding to CD112 (Nectin-2) and CD155 (PVR) was measured by flow cytometry. Binding of KIR2DL3, KIR2DL5, TIGIT, DNAM-1 and CD96 to biotin (neg. control), CD112 and CD155 was assessed as MFI in three independent experiments ($n = 3$). The mean values of the experiments are shown as black bars and standard deviations are depicted as error bars.

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surface of the KIR2DL5ζ reporter cells. Reporter cell lines transfected with KIR2DL1ζ, KIR2DL3ζ and KIR3DL1ζ were used as controls. KIRζ reporter cells were co-incubated with CD155-coated beads as well as with anti-KIR- (pos. control), biotin- (neg. control) and CD112-coated beads. All tested reporter cell lines (KIR2DL1ζ, KIR2DL3ζ, KIR2DL5ζ and KIR3DL1ζ) upregulated CD69 expression upon co-incubation with their respective anti-KIR antibody (pos. control beads; compared to unstimulated controls) and showed no functional response to biotin-coated beads (neg. control beads) (Fig 2A and 2B). Only KIR2DL5ζ reporter cells showed a significant upregulation of CD69 expression upon incubation with CD155-coated beads compared to the co-incubation with negative control beads (biotin) or beads coated with CD112 ($p < 0.01$) (Figs 2A, 2B and S2). Blocking experiments using antibodies directed against KIR2DL5 or a respective isotype control were performed. The isotype antibody did not influence reporter cell activation following co-incubation with anti-KIR2DL5- and CD155-coated beads, whereas the anti-KIR2DL5 antibody significantly abrogated CD69 upregulation on KIR2DL5ζ reporter cells ($p < 0.05$) (Fig 2C). Taken together, these data demonstrate that the interaction between KIR2DL5 and CD155 results in the functional activation of KIR2DL5ζ reporter cells.

KIR2DL5$^+$ primary human NK cells are inhibited by CD155-expressing target cells

To determine the consequences of interactions between KIR2DL5 and CD155 for primary human KIR2DL5$^+$ NK cell function, NK cell degranulation upon co-incubation with 721.221

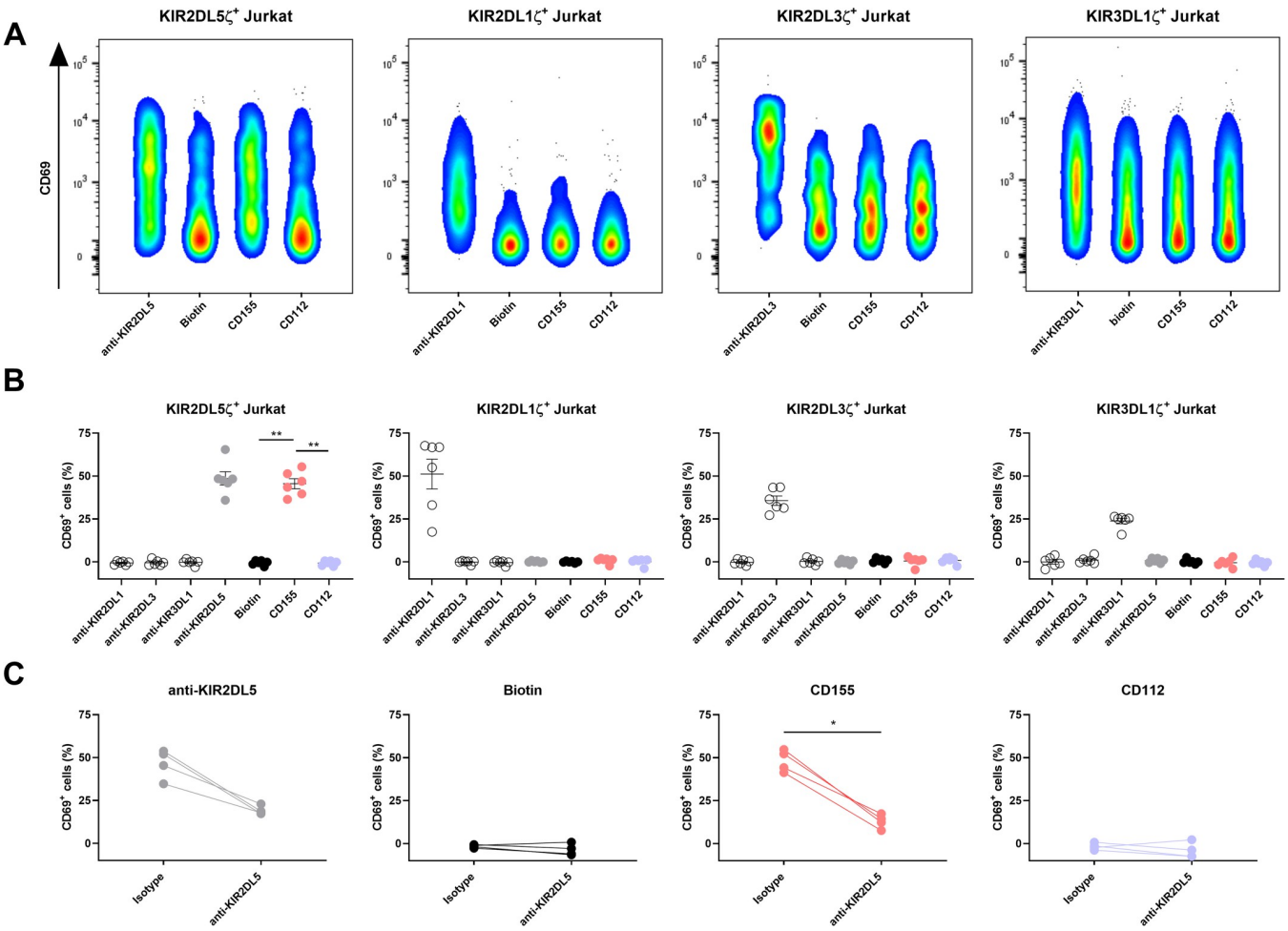

Fig 2. KIR2DL5ζ-expressing reporter cells are activated by interacting with CD155. (A) Reporter cell activity was determined by the upregulation of CD69 on the surface of KIR2DL1ζ, KIR2DL3ζ, KIR2DL5ζ and KIR3DL1ζ reporter cells during co-incubation with beads coated with anti-KIR2DL1, anti-KIR2DL3, anti-KIR2DL5 or anti-KIR3DL1, respectively, (pos. controls), biotin (neg. control), CD112 or CD155. Plots represent one out of six experiments and only the respective anti-KIR positive control is shown. (B) Percentage of CD69$^+$ reporter cells after co-incubation with the indicated antibodies, biotin and nectin(-like) molecules. Plots show the results of six independent experiments ($n = 6$) with the mean value for each condition (black bar) and error bars depicting the standard error of the mean. Background activation (no target control) was subtracted from all samples. Mann-Whitney test was used to statistically analyze the difference in CD69 expression of CD155-, CD112- and biotin-stimulated cells ($p = 0.0022$). (C) Reporter cell activity of KIR2DL5ζ cells was assessed after incubating cells with purified anti-KIR2DL5 or mouse IgG isotype control antibody prior to co-incubation with anti-KIR2DL5-, biotin-, CD155- and CD112-coated beads. The percentage of CD69$^+$ cells following incubation without beads (no target control) was subtracted from all samples. Reporter cell activity was determined in four independent experiments ($n = 4$). Lines between dots connect the matching samples incubated with the IgG isotype antibody or with purified anti-KIR2DL5. Mann-Whitney test was used to calculate statistical significance of differences in reporter cell activation through co-incubation with CD155-coated beads with and without KIR2DL5 blocking antibody ($p = 0.0286$).

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cells, which do not express CD155 at the cell surface, and transduced 721.221 cells overexpressing CD155 (S3 Fig), was assessed. NK cells of a *KIR2DL5A*001*-positive donor and a donor completely lacking KIR2DL5 genetically were co-cultured with CD155$^+$ or CD155$^-$ target cells, and CD107a expression levels on KIR2DL5$^-$ and KIR2DL5$^+$ NK cells served as readout for NK cell degranulation in response to the different target cells (Fig 3A). All NK cell populations (KIR2DL5$^-$, KIR2DL5$^+$ and NK cells from *KIR2DL5*-negative donors) showed increased CD107a expression after co-incubation with CD155$^-$ 721.221 cells (Fig 3B). When co-incubating NK cells with CD155$^+$ 721.221 cells, KIR2DL5$^+$ NK cells expressed significantly lower CD107a levels compared to KIR2DL5$^-$ NK cells ($p < 0.01$) and NK cells of *KIR2DL5*-negative

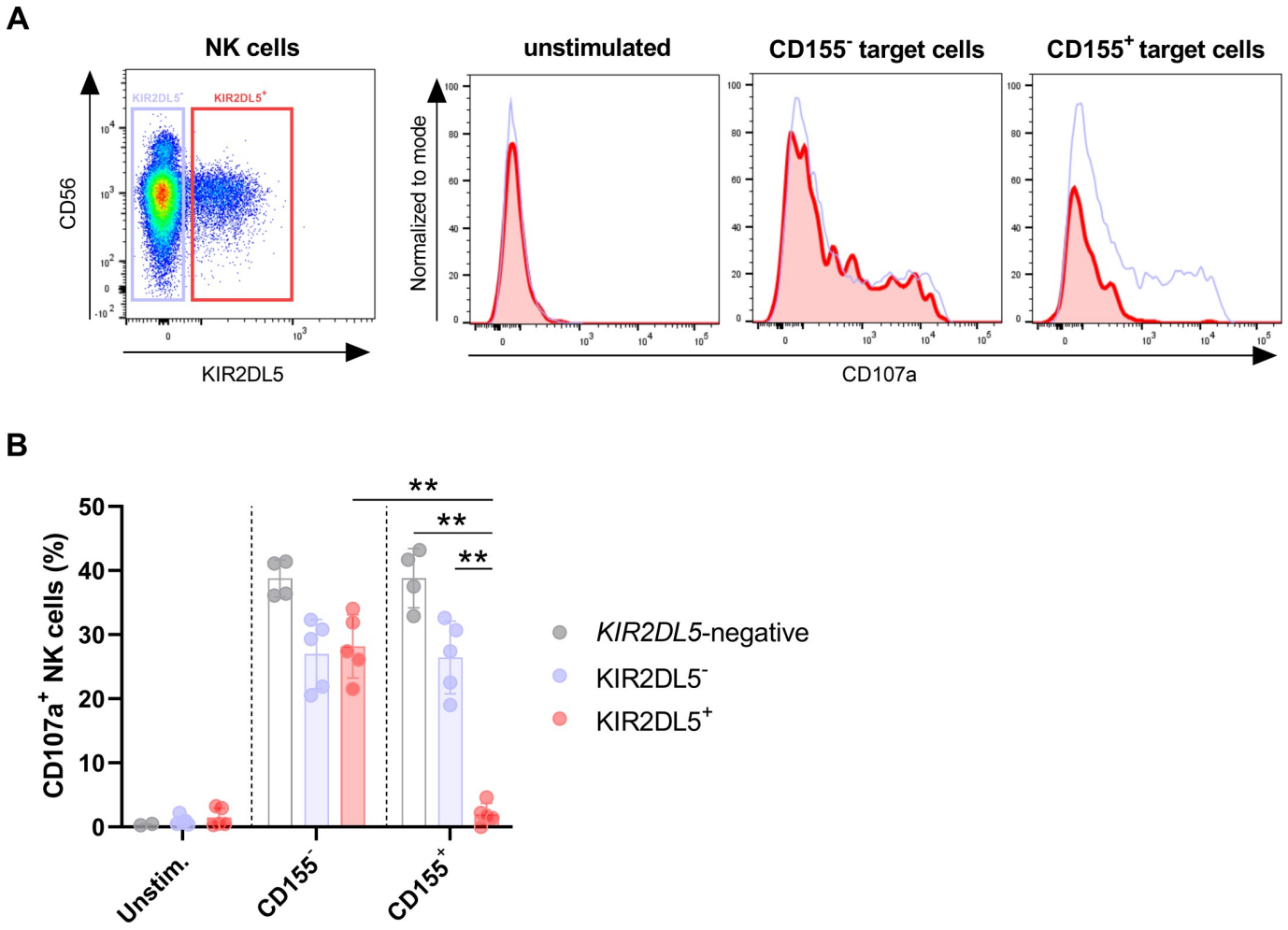

Fig 3. Primary human KIR2DL5$^+$ NK cells are inhibited by CD155-expressing target cells. (A) Degranulation of KIR2DL5$^+$ (red) and KIR2DL5$^-$ (blue) NK cells was defined as percentage of CD107a$^+$ NK cells after co-incubation with CD155$^-$ 721.221 target cells, CD155$^+$ 721.221 target cells or left unstimulated. Plots show one representative experiment out of five independent experiments. (B) Percentage of CD107a-positive NK cells after co-incubation with target cells is shown as indicated before. Bars indicate mean values with standard deviations of KIR2DL5$^+$ donors (n = 5 from 5 donors) and *KIR2DL5*-negative donors (n = 4 from 2 donors). Mann-Whitney test was used to calculate statistical significance of differences in CD107a levels (KIR2DL5$^-$ versus KIR2DL5$^+$ NK cells p = 0.008; *KIR2DL5*-negative versus KIR2DL5$^+$ NK cells p = 0.016; KIR2DL5$^+$ NK cells co-cultured with CD155$^+$ or CD155$^-$ target cells p = 0.008).

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donors ($p < 0.02$), and compared to KIR2DL5$^+$ NK cells co-cultured with CD155$^-$ target cells ($p < 0.01$) (Fig 3A and 3B). Taken together, these data show that interactions between CD155 and KIR2DL5 result in functional inhibition of primary human KIR2DL5$^+$ NK cells.

HIV-1 mediates Nef-dependent downregulation of CD155 on HIV-1-infected CD4$^+$ T cells

HIV-1 has developed several mechanisms to evade innate and adaptive immune responses by modulating the expression of cell surface molecules such as HLA class I [14,15,31]. Previous studies have suggested that HIV-1 can also modulate protein expression levels of CD155 on the surface of infected cells. However, CD155 expression in HIV-1 infection is under debate, as some studies described a downregulation [22–25] whereas others reported no effect or an upregulation [20,26,27]. While most previous studies used tumor-transformed cell lines as infection models and/or pseudotyped HIV-1 particles, we decided to determine how genuine

HIV-1 strains, including primary transmitted-founder HIV-1 infectious molecular clones (IMCs), impact CD155 expression on primary CD4$^+$ T cells. Freshly isolated human CD4$^+$ T cells were infected with cell line-adapted (NL4-3 and JR-CSF) or primary (CH077, CH164, CH185, CH198, CH236, CH293) HIV-1 strains and median fluorescence intensity (MFI) levels of HLA class I (HLA-I), HLA-E, tetherin and CD155 were compared between HIV-1-infected p24 Gag$^+$ CD4^{dim} cells and uninfected p24 Gag$^-$ CD4$^+$ cells within the same experimental tube (Figs 4A and S4). In line with previous studies [31–33], CD4$^+$ T cells infected with different HIV-1 strains exhibited lower expression levels of HLA-I, HLA-E and tetherin than uninfected cells, indicating downregulation of these molecules by HIV-1 (exemplarily shown for the HIV-1 strain CH077 in Fig 4A). Similarly, the expression levels of CD155 were significantly decreased on HIV-1-infected cells compared to uninfected CD4$^+$ T cells, and this downregulation was observed for all investigated laboratory-adapted and primary HIV-1 strains (Fig 4B). CD155 levels on uninfected bystander cells were similar to those on mock-infected CD4$^+$ T cells of the same donor, which were treated the same but without adding virus (Fig 4A and 4B). CD155 levels depicted as relative change for the different HIV-1 strains are shown in Fig 4B and revealed the strongest downregulation of CD155 on CD4$^+$ T cells infected with CH077, CH198 and NL4-3. Taken together, laboratory-adapted and primary HIV-1 strains downmodulate CD155 from the surface of primary human HIV-1-infected CD4$^+$ T cells.

The HIV-1 accessory protein Nef is known to mediate downregulation of HLA-I cell surface expression [31], and was previously also suggested to be involved in CD155 modulation [22,24]. To investigate whether Nef contributes to the modulation of CD155 by CH077, CH198 and NL4-3, which showed the strongest downregulation of CD155, we used the respective wild type (wt) and Nef-defective mutant (ΔNef) viruses to infect CD4$^+$ T cells. As Nef also targets CD4 surface expression, ΔNef-infected CD4$^+$ T cells were defined as p24 Gag$^+$ tetherin$^-$ CD4$^+$ T cells. All three tested HIV-1 wt strains induced downregulation of HLA-ABC on the surface of infected CD4$^+$ T cells, which was not observed or less pronounced when infecting cells with the respective ΔNef mutant viruses (Fig 4C). While the wild type viruses also downmodulated CD155 as described above, the ΔNef mutants did not downregulate CD155 expression to the same extent ($p < 0.01$) (Fig 4C). These data demonstrate that the accessory HIV-1 protein Nef is involved in the downregulation of CD155 from the surface of infected primary human CD4$^+$ T cells.

Reduced *in vitro* inhibition of HIV-1 replication by KIR2DL5$^+$ NK cells against Nef-deficient strains

To evaluate the consequences of CD155-downregulation for the antiviral capacity of KIR2DL5$^+$ NK cells, we co-incubated HIV-1 CH198 and NL4-3-infected (wt and ΔNef) CD4$^+$ T cells with autologous KIR2DL5$^+$ or KIR2DL5$^-$ NK cell clones. While KIR2DL5$^+$ and KIR2DL5$^-$ NK cell clones differed significantly in the expression of KIR2DL5 (KIR2DL5 median fluorescence intensity (MFI) for KIR2DL5$^+$ NK cell clones: 400; KIR2DL5 MFI for KIR2DL5$^-$ NK cell clones: 8; $p < 0.0001$), expression of TIGIT, DNAM-1 and CD96 was similar between the clones ($p > 0.1$). After a co-incubation of 7 days, viral inhibition was assessed by quantifying the percentage of p24$^+$ cells. KIR2DL5$^+$ NK cells exhibited a significantly higher inhibition of viral replication of CH198 and NL4-3 wt viruses compared to the respective ΔNef viruses ($p < 0.01$), while only a small effect (NL4-3) or no effect (CH198) was observed for KIR2DL5$^-$ NK cells (Fig 5A and 5B). These data indicate that the ability of KIR2DL5$^+$ NK cells to inhibit HIV-1 replication *in vitro* is significantly affected by Nef-mediated regulation of CD155, while the ability of KIR2DL5$^-$ NK cells is not. This was further supported by the observation that inhibition of replication of ΔNef CH198 (lacking the ability to downregulate

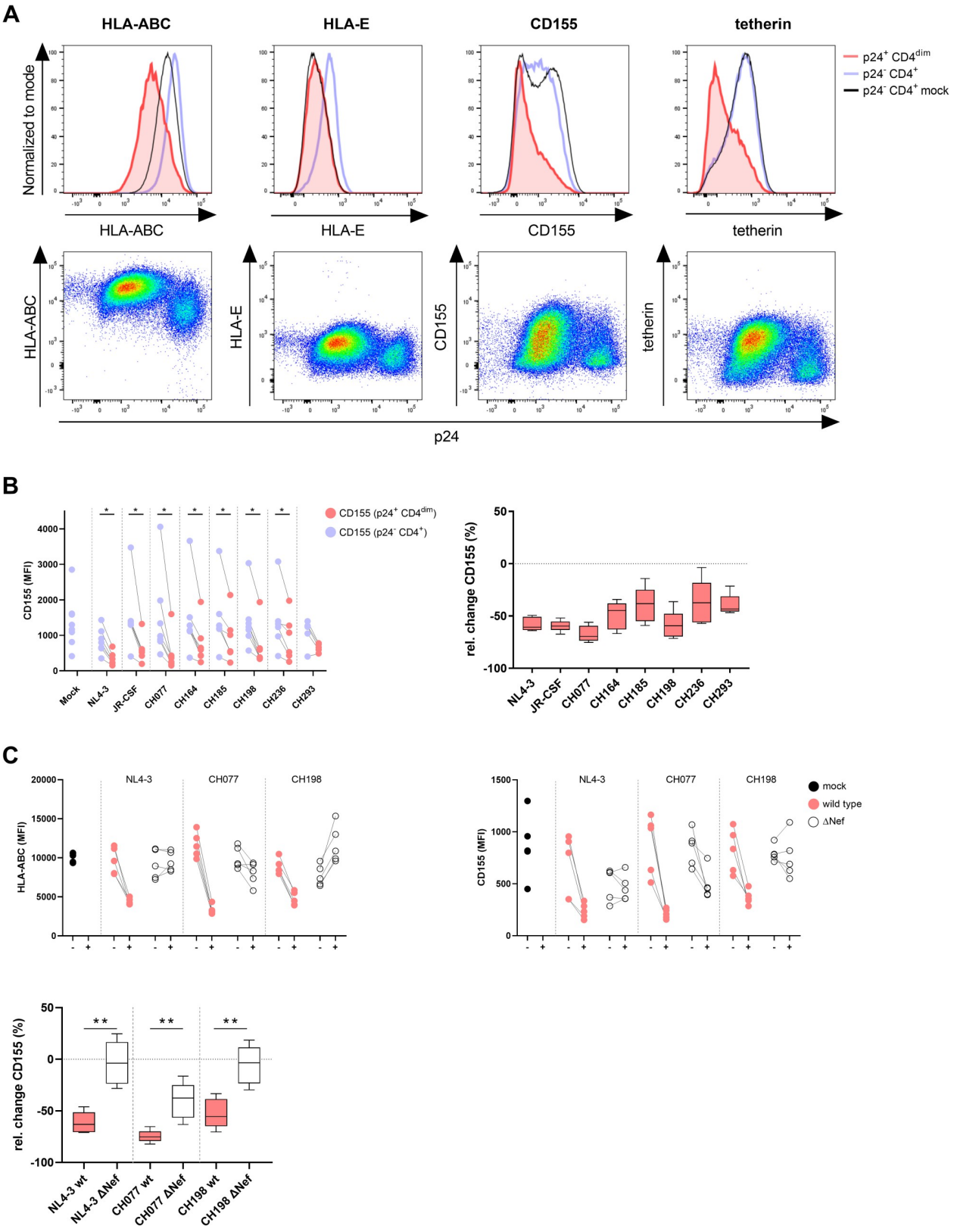

Fig 4. HIV-1 mediates downregulation of CD155 on infected CD4$^+$ T cells in a Nef-dependent manner. (A) Expression levels of HLA-ABC, HLA-E, CD155 and tetherin were compared between HIV-1-infected (CH077) and uninfected CD4$^+$ T cells. HIV-1-infected cells were determined by gating on p24$^+$ CD4^{dim} cells and uninfected cells were defined as p24$^-$ and CD4$^+$. Histograms show surface expression of the indicated molecules

on HIV-1-infected (red), uninfected (blue) and mock infected (black) CD4$^+$ T cells. Dot plots display surface expression of HLA-ABC, HLA-E, CD155 and tetherin on p24$^-$ and p24$^+$ CD3$^+$ T cells. (B) Modulation of CD155 expression on HIV-1-infected CD4$^+$ T cells was assessed by using different HIV-1 cell line-adapted (NL4-3, JR-CSF) and primary strains (CH077, CH164, CH185, CH198, CH236, CH293). Mock-infected cells served as a control. The left graph shows CD155 expression on uninfected (blue) compared to infected (red) cells depicted as MFI. Each dot represents the result of one independent experiment. The right graph shows the median relative change (%) of CD155 expression mediated by the different HIV-1 strains, which was calculated as (MFI infected–MFI uninfected)/MFI uninfected × 100. HIV-1 strains (NL4-3, JR-CSF, CH077, CH164, CH185, CH198 and CH236) showed a significant downregulation of CD155 on infected cells (NL4-3, CH077, CH164, CH198 p = 0.016; JR-CSF, CH185, CH236 p = 0.03), calculated by performing the Wilcoxon matched-pairs signed rank test. Samples <150 cells were excluded, resulting in varying numbers of donors per condition. (C) HIV-1 wild type (wt) strains that showed the strongest CD155 downregulation (NL4-3, CH077, CH198) were compared to ΔNef mutant viruses and analyzed for their ability to downmodulate HLA-ABC and CD155. Expression levels depicted as MFI are shown for mock (black), wt virus (red) and ΔNef virus (white) infected CD4$^+$ T cells (n = 5). The median relative change of CD155 expression is shown for wt (red) and ΔNef mutant viruses (white). Mann-Whitney test was used to calculate statistical significance of differences between the downregulation of CD155 by HIV-1 wt and ΔNef mutant viruses (p = 0.008).

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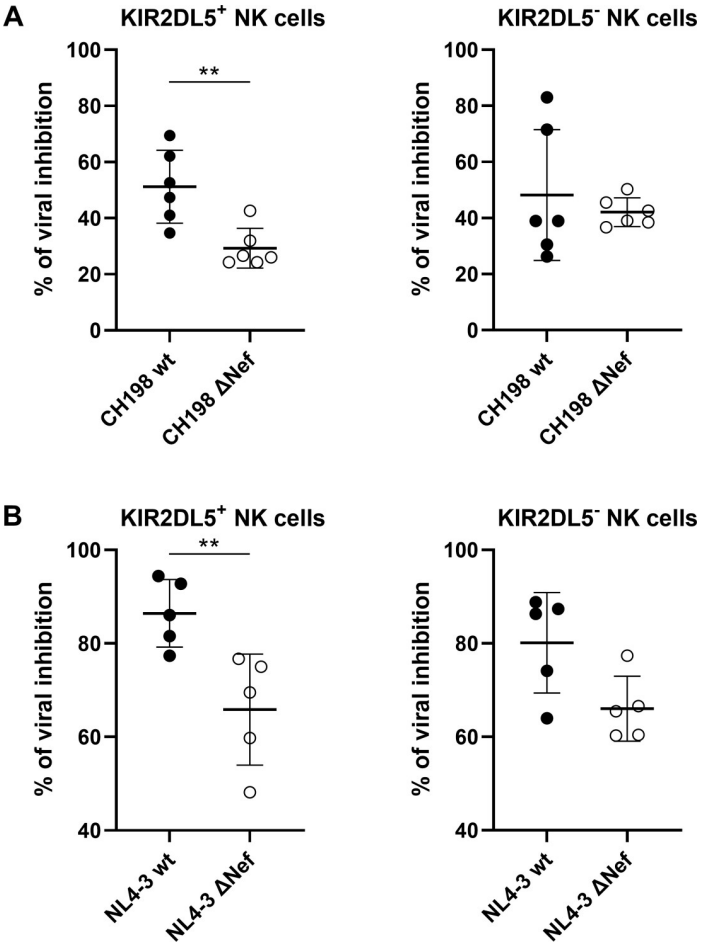

Fig 5. Inhibition of HIV-1 replication by KIR2DL5$^+$ and KIR2DL5$^-$ NK cells. Anti-viral activity of KIR2DL5$^+$ and KIR2DL5$^-$ NK cells was evaluated by co-culture of KIR2DL5$^+$ and KIR2DL5$^-$ NK cell clones with HIV-1 CH198 (A) and NL4-3 (B) wt and ΔNef virus infected autologous CD4$^+$ T cells. Graphs show the percentage of viral inhibition based on the percentage of p24$^+$ CD4$^+$ T cells in co-culture with NK cells compared to the CD4$^+$ T cell only control where no NK cells were added. Viral inhibition was calculated as 100*(1-(percentage of p24$^+$ CD4$^+$ T cells with NK cells / percentage of p24$^+$ CD4$^+$ T cells without NK cells)). Mann-Whitney test was used to calculate statistical significance of differences in viral inhibition between wt and ΔNef viruses (CH198: p = 0.009; NL4-3: p = 0.008). Data were obtained by using six (for CH198 viral inhibition) and five (for NL4-3 viral inhibition) KIR2DL5$^+$ and six/five KIR2DL5$^-$ NK cell clones from two donors (CH198: *n* = 6; NL4-3: *n* = 5) and results are shown as mean values with SD.

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CD155) was significantly lower by KIR2DL5$^+$ NK cells compared to KIR2DL5$^-$ NK cells ($p < 0.02$) (Fig 5A). Inhibition of replication of ΔNef NL4-3 by NK cells was more variable (Fig 5B). Taken together, these data demonstrate a significant impact of changes in CD155 expression observed between wild type and ΔNef strains for the ability of KIR2DL5$^+$ NK cells to inhibit viral replication *in vitro*.

Discussion

The different members of the KIR family contribute to the tight regulation of NK cell function by mediating inhibitory or activating signals through interactions with HLA class I. The inhibitory NK cell receptor KIR2DL5 is the most recently identified human KIR for which a ligand was not identified for almost two decades [6,34]. Due to the fact that ligands remained unknown, the functional relevance of KIR2DL5-expressing NK cells in human diseases is poorly understood. Recently, the nectin-like molecule CD155 was reported to interact with KIR2DL5 [8,9], making KIR2DL5 the first KIR that binds to other proteins than HLA class I molecules. Here, we demonstrate that CD155 serves as a functional ligand of KIR2DL5, mediating inhibitory signals resulting in decreased activity of primary KIR2DL5$^+$ NK cells against CD155-expressing target cells. Furthermore, we show that the viral protein Nef decreases CD155 expression levels on HIV-1-infected primary human CD4$^+$ T cells, leading to better inhibition of the *in vitro* replication of wild type HIV-1 strains compared to Nef-deficient strains by KIR2DL5$^+$ NK cells. Taken together, these data demonstrate that KIR2DL5 is an important binding partner of CD155, modulating KIR2DL5$^+$ NK cell-mediated immune responses against HIV-1-infected target cells.

The family of nectin and nectin-like molecules has been identified as a group of ligands for inhibitory and activating NK cell receptors, thereby contributing to the regulation of NK cell function [30]. CD155 is a member of the immunoglobulin superfamily and has central roles in cell adhesion and immune responses [30]. While the binding of CD155 to the activating NK cell receptors DNAM-1 [10] and CD96 [11] as well as to the inhibitory receptor TIGIT [12] is well established, the here described functional interaction between CD155 and the inhibitory NK cell receptor KIR2DL5 adds another axis to the complex regulation of NK cells by CD155. KIRs are known to interact with several of the highly polymorphic HLA class I molecules on the surface of normal cells, keeping NK cell effector functions tightly regulated. In contrast to other KIRs, including the structural closely related KIR2DL4 [6,35], KIR2DL5 is the first KIR for which no strong binding to any of the tested HLA molecules has been observed, indicating that HLA class I and class II might not represent the main binding partners for KIR2DL5. KIR2DL5 genes are present in all human populations with frequencies ranging between 26% and 86%, and are expressed on up to 10% of total NK cells in healthy individuals encoding for *KIR2DL5* [6]. Like other KIRs, KIR2DL5 is genetically polymorphic, and due to a duplication of the gene in humans, encoded by two gene loci (*KIR2DL5A* and *KIR2DL5B*). The fact that only a minority of allotypes, mainly KIR2DL5A allotypes and most prevalent KIR2DL5A*001, have been shown to be expressed on the cell surface [6,7] may explain the interaction of a receptor encoded by a polymorphic gene with a conserved ligand such as CD155. However, binding of other KIRs to HLA class I can be modulated by specific peptides loaded to HLA class I [36–39], and it is therefore possible that KIR2DL5 might bind to HLA molecules under specific conditions. Previously, KIR2DL5 was suggested to mediate inhibitory signals [7,8,40]; however, due to the lack of a well-defined cellular ligand the functional role of KIR2DL5 in the settings of infection or inflammation remained poorly understood. Here, we show inhibition of primary KIR2DL5$^+$ NK cells mediated through the CD155-KIR2DL5 axis using KIR2DL5$^+$ NK cells isolated from donors encoding for *KIR2DL5A*. The establishment of KIR2DL5 as an

inhibitory receptor binding CD155, although to a lesser extent than DNAM-1 and TIGIT, emphasizes the complex regulation of NK cell function by nectin and nectin-like molecules during health and disease.

Many viruses avoid recognition by immune cells through the modulation of surface expression of activating ligands. HIV-1 evades NK cell- and T cell-mediated immune responses through several mechanisms, including the downregulation of HLA class I molecules and stress-induced ligands on the surface of infected cells [13,14,16,19,20]. Here, we show that HIV-1 strains are also capable of downregulating CD155 on the surface of infected CD4$^+$ T cells, as previously reported [22–25]. In addition to the shown effects of Nef on CD155 expression, other studies have suggested an impact of Vpu on the downregulation of CD155 [22–24]. However, a Vpu-mediated regulation of CD155 remains controversial, as some studies described an involvement of Vpu [22,23] and some did not [27]. Furthermore, it has been shown that while Nef reliably downregulates CD155, the impact of Vpu varied between different HIV-1 strains [24]. CD155 serves as a binding partner of the activating NK cell receptor DNAM-1 [10]. Thus, downregulation of CD155 by HIV-1 has probably evolved in an effort to avoid DNAM-1-mediated NK cell recognition. The newly described interaction between the inhibitory KIR2DL5 and CD155 therefore has functional consequences for KIR2DL5$^+$ NK cell responses against HIV-1. Due to the Nef-dependent downregulation of CD155 by HIV-1, infected cells became more vulnerable to recognition by KIR2DL5$^+$ NK cells, which resulted in enhanced inhibition of replication of HIV-1 wt strains compared to HIV-1 ΔNef strains by KIR2DL5$^+$ NK cells. Although it has been described that Nef-defective HIV-1 strains might have an overall lower replication capacity [41–45], KIR2DL5$^+$ NK cells showed a significantly decreased inhibition of replication of Nef-deficient CH198 IMCs compared to KIR2DL5$^-$ NK cells, indicating a KIR2DL5-dependent effect. Individuals encoding for the expressed *KIR2DL5A* gene might therefore have an advantage in controlling HIV-1 replication. A recent study revealed an association between KIR2DL5 and reduced mother-to-child transmission of HIV-1 in infants born by HIV-1-infected mothers [46], supporting a protective role for KIR2DL5. Given the limited expression of KIR2DL5 on the cell surface, future studies need to discriminate between *KIR2DL5A* and *KIR2DL5B* genotypes when studying implications of KIR2DL5 expression on transmission and disease outcomes, and investigate this in the context of TIGIT, as it has been reported that TIGIT expression is upregulated on NK cells during HIV-1-infection [47,48]. Furthermore, KIR2DL5 has been suggested to be beneficial for the outcome of several other infectious diseases, and might also play a role in the outcome of cancer, as CD155 is known to be overexpressed in tumors [49–53].

In this study, we provide novel insights into the regulation of KIR2DL5$^+$ NK cell function by interactions with the newly described ligand CD155, revealing a novel checkpoint inhibitor target for immunotherapeutic approaches. Furthermore, we demonstrate that HIV-1-mediated downregulation of CD155 can result in enhanced *in vitro* inhibition of HIV-1 replication by KIR2DL5$^+$ NK cells. While the inhibitory receptor TIGIT, which is expressed on T and NK cells and also binds to CD155, is already investigated as a target in anti-tumor [54] and anti-HIV-1 [55] therapies, the inhibitory interactions between CD155 and KIR2DL5 might represent a second axis that can be targeted by future therapeutic approaches.

Materials and methods

Ethics statement

Peripheral blood samples were obtained from healthy blood donors 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). All participants were adults.

Primary cells and cell lines

Primary human peripheral blood mononuclear cells (PBMCs) were isolated from whole blood donations from healthy donors by performing density-gradient centrifugation. Isolated PBMCs were washed and cultured in complete R10 medium (RPMI-1640 medium (Sigma) supplemented with 10% fetal bovine serum (FBS, Sigma)). PBMCs were directly used for experiments or cell populations such as NK cells and CD4$^+$ T cells were isolated as indicated in detail below. Enriched NK cells were cultured overnight in complete medium supplemented with 1 ng/ml IL-15 before they were used in functional assays. HEK293T cells (American Type Culture Collection (ATCC), Cat#CRL-11268) were cultured in Dulbecco's Modified Eagle Medium (DMEM (Life Technologies)) supplemented with 10% FBS (D10). Jurkat E6.1 (ATCC, Cat#TIB-152), B-LCL 721.221 (RRID: CVCL_6263) [56] and RPMI-8866 (RRID:CVCL_1668) cells were maintained in complete R10 medium. Jurkat reporter cells are derived from the Jurkat clone E6.1 and lack the expression of β_2-microglobulin (β_2m-KO) [57]. Cells were engineered to express KIRζ chimeric constructs as previously described [57]. The KIR2DL1ζ, KIR2DL3ζ and KIR3DL1ζ cells were generated previously [36,57]. KIR2DL5ζ constructs were generated by fusing the extracellular and transmembrane domain of KIR2DL5 to the intracellular CD3ζ chain. Constructs for Jurkat reporter cells and constructs for generating CD155-expressing 721.221 cells were obtained from GeneArt GeneSynthesis (Thermo Fisher) and cloned into a lentiviral transfer vector encoding for a puromycin resistance. To generate Jurkat reporter cells and 721.221 cells expressing the gene of interest, cells were lentivirally transduced. To this end, HEK293T cells were transfected with a VSV-G envelope vector (pHEF-VSVG; NIH AIDS Reagent Program), an HIV-1 Gag-Pol packaging vector (psPAX2; NIH AIDS Reagent Program) and the transfer vector (p SFFV-IRES-Puro (pSIP)-ZsGreen) carrying the gene of interest by using Lipofectamine 3000 (Life Technologies). Lentiviral supernatant was harvested after 48 hours and used to transduce β_2m-KO Jurkat cells and 721.221 cells. After 3 days, cells were selected in 1 μg/ml puromycin (Sigma-Aldrich). Cells were cultured in complete R10 medium and maintained with 1 μg/ml puromycin.

Recombinant human KIR-Fc construct binding to ligand-coated beads

Streptavidin Dynabeads (Thermo Fisher Scientific) were coated with either 200 pmol biotinylated protein or 10 μg biotinylated IgG/mg Dynabeads. Ligand-screening was performed by coating with biotinylated CD155 (PVR) (AcroBiosystems), CD112 (Nectin-2) (BPSBiosciences) or biotin as a negative control and anti-KIR2DL5 as a positive control. To screen for interactions with HLA class I and class II molecules, the LABScreen Single Antigen Class I and II kits (OneLambda) were used. Negative control beads were not coated with HLA antigens and positive control beads were coated with purified human IgG. Recombinant human Fc constructs (KIR2DL1-Fc, KIR2DL3-Fc, KIR2DL4-Fc, KIR2DL5-Fc, KIR3DL1-Fc, CD96-Fc, DNAM-1-Fc, LAG-3-Fc, PVR(CD155)-Fc, TIGIT-Fc) (R&D Systems) were diluted to 250 μg/ml in PBS and co-incubated with coated beads for 15 min at 4˚C at a final concentration of 25 μg/ml. Samples were washed and bead-bound Fc constructs were detected by a staining with F(ab)2 goat-anti-human IgG PE secondary antibody (Life Technologies) for 15 min at 4˚C. Interactions between Fc construct and peptide-coated beads were either quantified by flow cytometry (LSR Fortessa (BD Biosciences)) or by using the Luminex xMAP technology on a Bio-Plex 200 (Bio-Rad Laboratories).

KIRζ reporter cell assay

Streptavidin Dynabeads were coated with biotinylated PVR (CD155), Nectin-2 (CD112), biotin, anti-KIR2DL1, anti-KIR2DL3, anti-KIR2DL5 and anti-KIR3DL1 as described before. 2.5 x 10^4 cells of each reporter cell line were seeded into a well of a tissue culture-treated 96-well plate and co-incubated with 10 μl of the protein-coated beads for 5 h at 37˚C/5% CO_2 in a final volume of 200 μl. For blocking experiments, prior to co-incubation with beads, KIR2DL5$^+$ JRC were blocked for 30 min with 30 μg/ml purified anti-KIR2DL5 or 30 μg/ml purified IgG1 isotype control antibody. Blocking antibodies remained in the wells during the whole assay. After co-incubation, cells were washed with PBS and stained with the viability dye LIVE/DEAD Fixable Near-IR (Life Technologies), anti-CD3-BUV395 (clone UCHT1, BD Biosciences), anti-CD69-BV421 (clone FN50, Biolegend) and the appropriate KIR antibody conjugated to PE (anti-KIR2DL1-PE (clone REA284), anti-KIR2DL3-PE (clone REA147), anti-KIR2DL5-PE (clone UP-R1), anti-KIR3DL1-PE (clone DX9) (Miltenyi). Cells were fixed in CellFix (BD Biosciences) and CD69 expression as a readout for KIR crosslinking was analyzed by flow cytometry.

NK cell degranulation assay

NK cell degranulation upon co-incubation with target cells was determined by the expression of CD107a on the cell surface, which serves as a surrogate marker for NK cell degranulation [58]. In brief, overnight cultured NK cells enriched with the EasySep human NK cell enrichment kit (StemCell Technologies) from PBMCs from *KIR2DL5A*001*-positive donors or donors lacking KIR2DL5 genetically were co-cultured with CD155-expressing (CD155-transduced 721.221) or CD155-nonexpressing (721.221) target cells at an effector to target ratio of 1:2 in 200 μl complete R10 for 4 h at 37˚C. During co-incubation, each well contained 2 μl anti-CD107a (clone LAMP-1, Biolegend) and 25μl/ml Brefeldin A. Cells were subsequently stained with LIVE/DEAD Fixable Near-IR and with the following antibodies: anti-CD3-BUV395 (clone UCHT1, BD), anti-CD16-PE-Cy7 (clone 3G8, Biolegend), anti-CD56-BV785 (clone NCAM16.1, BD), anti-KIR2DL5-PE (clone UP-R1, Biolegend) for 15 min at RT and fixed with CellFix (BD) before flow cytometric acquisition.

Enrichment and stimulation of primary human CD4$^+$ T cells

CD4$^+$ T cells were isolated from fresh PBMCs trough negative selection with the EasySep human CD4 T cell enrichment kit (StemCell Technologies) according to the manufacturer's protocol. After isolation, cells were cultured in complete R10 medium supplemented with 100U / ml IL-2 (Peprotech) and stimulated with anti-CD3/anti-CD28 Dynabeads (Life Technologies) for 3 days at 37˚C / 5% CO_2 at a bead to cell ratio of 1:2. Before infecting the stimulated cells, beads were washed out.

Generation of HIV-1 virus stock from infectious molecular clones

HIV-1 viral stocks were produced as described previously [32]. In brief, plasmids harboring the full length proviral genome of infectious molecular clones of the primary strains CH077, CH164, CH185, CH198, CH236, CH293 (kindly provided by the Beatrice Hahn and John Kappes Laboratories) and the cell line-adapted strains NL4-3 and JR-CSF (National Institutes of Health (NIH); catalog no. 114 and 2708) as well as the respective *Δnef* mutants, which were generated previously [32], were used to transfect HEK293T cells. Therefore, 24 μg of DNA was diluted in Opti-MEM for a Lipofectamine 3000 transfection (Life Technologies) of HEK293T

cells in a T75 flask according to manufacturer's protocol. Samples were filled up to 10 ml with fresh cell culture medium (D10) and lentiviral particles were harvested 48 h after transfection. The supernatant was centrifuged at 500 x g, filtered through a 0.45 nm filter and concentrated by using Lenti-X concentrator (Clontech Labs). Viral stocks were aliquoted and stored at -80˚C until further use.

Infection of primary human CD4$^+$ T cells with HIV-1 viral stocks

Stimulated CD4$^+$ T cells were resuspended in the respective HIV-1 viral stock (Table 1) or in cell culture medium (mock control) and cells were spinfected for 2 h at 1.200 x g and 37˚C. After spinfection, viral supernatant was removed and fresh complete medium (R10) supplemented with 100 U IL-2/ml was added to the cells. The infected cells were incubated for 72 h at 37˚C/5% CO_2 until antibody stainings for flow cytometry analysis took place. CD4$^+$ T cells used for long-term co-culture with NK cell clones were infected for 4 h at 37˚C without centrifugation to increase cell viability.

Flow cytometry analysis of surface markers and intracellular staining of HIV-1-infected CD4$^+$ T cells

To assess cell surface expression of proteins, flow cytometry was performed. Cells were washed with PBS and subsequently stained with the viability dye LIVE/DEAD Fixable Near-IR (Life Technologies) and with the antibodies anti-CD3-BUV395 (clone UCHT1, BD), anti-CD4-BV711 (clone RPA-T4, Biolegend), anti-CD155-PE (clone SKIL4, Biolegend), anti-HLA-ABC-Pe-Cy7 (clone W6/32, Biolegend), anti-HLA-E-BV421 (clone 3D12, Biolegend), anti-tetherin-APC (clone RS38E, Biolegend) and anti-IgG1-PE isotype control (clone MOPC21, Biolegend) for 15 min at RT. After washing the cells with PBS, an intracellular staining was performed. In brief, cells were incubated in BD Cytofix/Cytoperm for 20 min at 4˚C, washed with BD Perm/Wash buffer and stained with anti-p24-FITC (clone KC57, Beckman Coulter) for 20 min. After another washing step, cells were fixed in BD Cellfix and analyzed by flow cytometry (BD LSR Fortessa). HIV-1-infected cells were defined as p24 Gag$^+$ CD4^{dim} and uninfected as p24 Gag$^-$ CD4$^+$ cells. Cells infected with HIV-1 ΔNef mutant viruses were defined as p24 Gag$^+$ and tetherin$^-$ cell, as Nef-deficient viruses are not able to downregulate CD4.

Generation of NK cell clones

NK cell clones were generated as described previously [63]. In brief, NK cells were enriched from PBMCs isolated from a *KIR2DL5A*001*-positive healthy donor and were subcloned by fluorescence activated cell sorting (FACS). Single NK cells, either expressing or not expressing

Table 1. HIV-1 strains used in this study.

Group	Virus	Subtype	Source (reference)
Cell line-adapted strains	NL4-3	B	Infectious molecular clone [59]
	JR-CSF	B	Infectious molecular clone
Primary strains	CH077	B	Infectious molecular clone (founder) [60]
	CH164	C	Infectious molecular clone (founder) [61]
	CH185	C	Infectious molecular clone (founder) [61]
	CH198	C	Infectious molecular clone (founder) [61]
	CH236	C	Infectious molecular clone (founder) [62]
	CH293	C	Infectious molecular clone (chronic) [61]

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KIR2DL5, were sorted into 96-well plates and cultured for 14 days in the presents of irradiated allogenic PBMCs and 8866 feeder cells at a ratio of 10:1 in NK cell cloning medium supplemented with 1 μg/ml phytohaemagglutinin (PHA (Invitrogen)). Cloning medium consisted of RPMI medium supplemented with 5% human serum (Sigma-Aldrich), 10% FBS (Sigma-Aldrich), 1X MEM-NEAA (Gibco), 1X sodium pyruvate (Gibco), 1X (2 mM) L-glutamine (Sigma-Aldrich), 100 μg/mL Primocin (Invivogen) and 200 U/mL IL-2 (Roche). After 14 days, outgrowing cells were transferred to a 48-well plate and cultured in NK cell cloning medium with frequent medium exchange and further expansion.

NK cell co-cultivation with autologous HIV-1-infected CD4$^+$ T cells

To analyze the antiviral potential of KIR2DL5$^+$ NK cells, CD4$^+$ T cells from the same donor from which NK cell clones were generated were stimulated and infected with HIV-1 CH198 or NL4-3, as described above. After 4 h of infection, CD4$^+$ T cells were co-incubated with autologous KIR2DL5$^+$ or KIR2DL5$^-$ NK cell clones at an effector to target ratio of 2:1 in 300 μl complete R10 medium supplemented with 100 U/ml IL-2 (Peprotech) for 7 days. 100 μl of cell culture supernatant was replaced by fresh medium every 2 days. After 7 days, antibody staining with anti-CD3, anti-CD4, anti-tetherin, anti-CD16, anti-CD56 and intracellular staining with anti-p24 was performed and cells were analyzed by flow cytometry.

Data analysis

Flow cytometry data were acquired on a BD LSR Fortessa (Biosciences) in the core facility

Flow Cytometry at the Leibniz Institute of Virology and analyzed using FlowJo software 10.7.1 (BD Biosciences). Data were statistically analyzed and graphically displayed in Graphpad Prism 9.0.1. Statistical analysis were performed using the non-parametric Mann-Whitney test or the Wilcoxon matched-pairs signed rank test for paired samples. If not indicated otherwise, mean values with standard deviations (SD) are shown for each group.

Supporting information

S1 Fig. KIR-Fc construct binding to CD155-coated beads. KIR-Fc construct binding to biotin, CD112 and CD155 measured by flow cytometry. Binding of KIR2DL1, KIR2DL4 or KIR3DL1 to biotin (neg. control), CD112 or CD155 was assessed as median fluorescence intensity (MFI) in three independent experiments ($n = 3$). The mean values of the experiments are shown as black bars and standard deviation is depicted as error bars.
(TIF)

S2 Fig. Gating strategy to define CD69 expression on KIR Jurkat reporter cells. Gating strategy for flow cytometric analyses of CD69 expression on Jurkat reporter cells. Jurkat cells were first defined by forward scatter area (FSC-A) and side scatter area (SSC-A) characteristics. After doublet exclusion using forward scatter area (FSC-A) and forward scatter height (FSC-H), viable cells were identified as negative for LIVE/DEAD Near-IR staining (viability dye). Subsequently, gating on CD69 for the different conditions was performed (exemplary shown for co-incubation of Jurkat cells with biotin-, anti-KIR2DL5- or CD155-coated beads).
(TIF)

S3 Fig. CD155 surface expression on 721.221 cells. Flow histogram shows expression levels of CD155 on the cell surface of parental 721.221 (CD155$^-$) (white) and transduced 721.221 (CD155$^+$) (gray) cells measured by using an anti-CD155 antibody.
(TIF)

S4 Fig. HIV-1-mediated downregulation of CD155. CD155 expression levels were compared between HIV-1-infected (red) and uninfected (blue) $CD4^+$ T cells. HIV-1-infected cells were determined by gating on $p24^+$ $CD4^{dim}$ cells and uninfected cells were defined as $p24^-$ and $CD4^+$. Histogram shows CD155 surface expression on HIV-1-infected (red), uninfected (blue) and mock-infected (black) $CD4^+$ T cells, including isotype controls (HIV^+ dashed black, HIV^- dashed grey).
(TIF)

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Author Contributions

Conceptualization: Pia Fittje, Angelique Hœlzemer, Wilfredo F. Garcia-Beltran, Marcus Altfeld.

Data curation: Marcus Altfeld.

Formal analysis: Pia Fittje.

Funding acquisition: Christian Körner, Marcus Altfeld.

Investigation: Pia Fittje, Sarah Vollmers, Annika Niehrs, Kerri Hagemann.

Methodology: Pia Fittje, Angelique Hœlzemer, Wilfredo F. Garcia-Beltran, Sarah Vollmers, Annika Niehrs, Glòria Martrus, Christian Körner.

Project administration: Marcus Altfeld.

Resources: Angelique Hœlzemer, Wilfredo F. Garcia-Beltran, Frank Kirchhoff, Daniel Sauter.

Supervision: Marcus Altfeld.

Validation: Pia Fittje, Angelique Hœlzemer, Glòria Martrus, Christian Körner, Marcus Altfeld.

Visualization: Pia Fittje.

Writing – original draft: Pia Fittje.

Writing – review & editing: Angelique Hœlzemer, Wilfredo F. Garcia-Beltran, Sarah Vollmers, Annika Niehrs, Kerri Hagemann, Glòria Martrus, Christian Körner, Frank Kirchhoff, Daniel Sauter, Marcus Altfeld.

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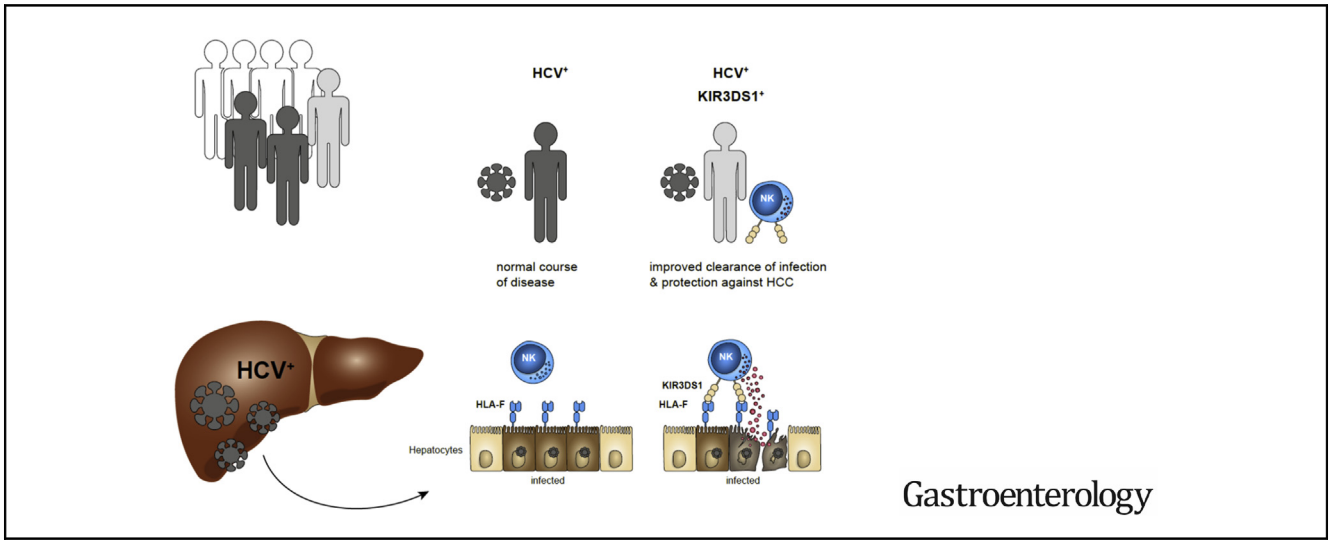
BRIEF REPORTS

Interactions Between KIR3DS1 and HLA-F Activate Natural Killer Cells to Control HCV Replication in Cell Culture

Sebastian Lunemann,[1] Anja Schöbel,[2] Janine Kah,[3] Pia Fittje,[1] Angelique Hölzemer,[1,4,5] Annika E. Langeneckert,[1] Leonard U. Hess,[1] Tobias Poch,[3] Gloria Martrus,[1,5] Wilfredo F. Garcia-Beltran,[6] Christian Körner,[1] Annerose E. Ziegler,[1] Laura Richert,[1,7] Karl J. Oldhafer,[8] Julian Schulze zur Wiesch,[4] Christoph Schramm,[3] Maura Dandri,[3] Eva Herker,[2] and Marcus Altfeld[1,5,9]

[1]Department of Virus Immunology, Heinrich Pette Institute, Leibniz Institute for Experimental Virology, Hamburg, Germany; [2]Junior Research Group HCV Replication, Heinrich Pette Institute, Leibniz Institute for Experimental Virology, Hamburg, Germany; [3]I. Department of Medicine, Center for Internal Medicine, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; [4]I. Department of Medicine, Section Infectious Diseases, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; [5]German Center for Infection Research (DZIF), Partner site Hamburg-Lübeck-Borstel-Riems, Hamburg, Germany; [6]Ragon Institute of MGH, MIT, and Harvard, Cambridge, Massachusetts: [7]INSERM U1219, INRIA SISTM, Bordeaux University, Bordeaux, France; [8]Department of General and Abdominal Surgery, Asklepios Hospital Barmbek, Semmelweis University of Medicine, Asklepios Campus, Hamburg, Germany; and [9]Institute for Immunology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany



Killer-cell immunoglobulin-like receptors (KIRs) are transmembrane glycoproteins expressed by natural killer (NK) cells. Binding of KIR3DS1 to its recently discovered ligand, HLA-F, activates NK cells and has been associated with resolution of hepatitis C virus (HCV) infection. We investigated the mechanisms by which KIR3DS1 contributes to the antiviral immune response. Using cell culture systems, mice with humanized livers, and primary liver tissue from HCV-infected individuals, we found that the KIR3DS1 ligand HLA-F is up-regulated on HCV-infected cells, and that interactions between KIR3DS1 and HLA-F contribute to NK cell–mediated control of HCV. Strategies to promote interaction between KIR3DS1 and HLA-F might be developed for treatment of infectious diseases and cancer.

Keywords: Antiviral Immune Response; Regulation; Immunity; Human Leukocyte Antigen.

More than 70 million people are infected with the hepatitis C virus (HCV) worldwide, and despite novel antiviral treatments, HCV remains one of the leading causes for end-stage liver diseases and transplantation. Natural killer (NK) cells have been demonstrated to be important for the early control of HCV infection.[1] Genetic studies have shown an association between KIR3DS1, an activating NK cell receptor, and HCV disease outcomes, including reduced development of hepatocellular

Abbreviations used in this paper: **HCV, hepatitis C virus; KIR, killer cell immunoglobulin-like receptor; NK cell, natural killer cell.**

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WHAT YOU NEED TO KNOW

BACKGROUND AND CONTEXT

Natural Killer (NK) cells have been associated with control of HCV infection, HCV treatment response and prevention of HCC in HCV patients.

NEW FINDINGS

HLA-F is upregulated upon HCV infection and subsequently sensed by KIR3DS1+ NK cells which, in turn, control HCV replication.

LIMITATIONS

This was a study with a small sample size. While previous genetic associations have confirmed protective effects in large cohorts, validation of molecular mechanisms in larger cohorts is still needed.

IMPACT

These studies provide a novel functional correlate for the described protective effects of KIR3DS1+ NK cells in HCV infection, and a rationale to harness the interaction between KIR3DS1 and HLA-F as a novel immunotherapeutic tool in infectious diseases and cancer.

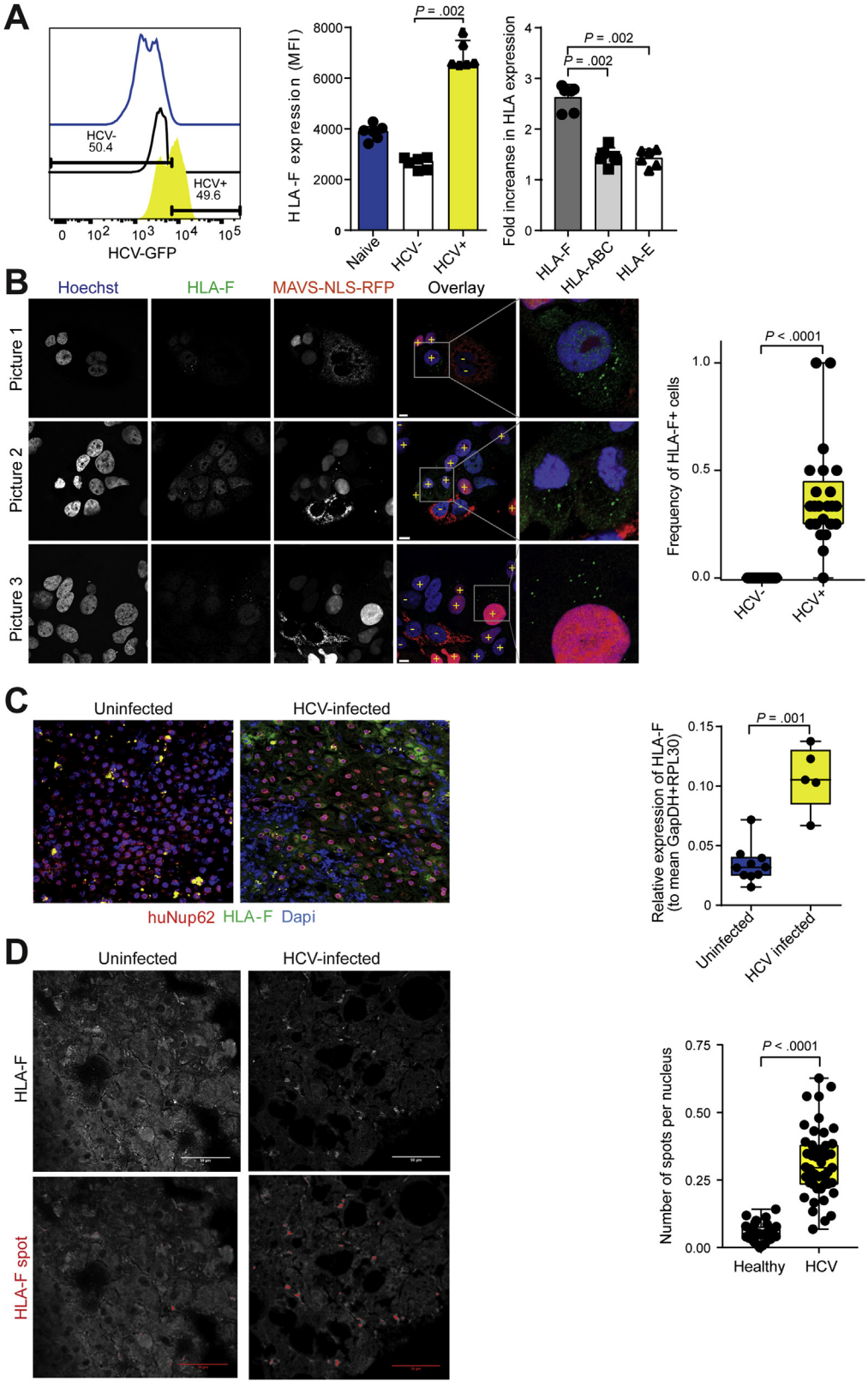
carcinoma.[2–4] KIR3DS1 recognizes open conformers of the nonclassical HLA class I molecule HLA-F.[5] Here we investigated the contribution of the interaction between KIR3DS1and HLA-F to the control of HCV replication.

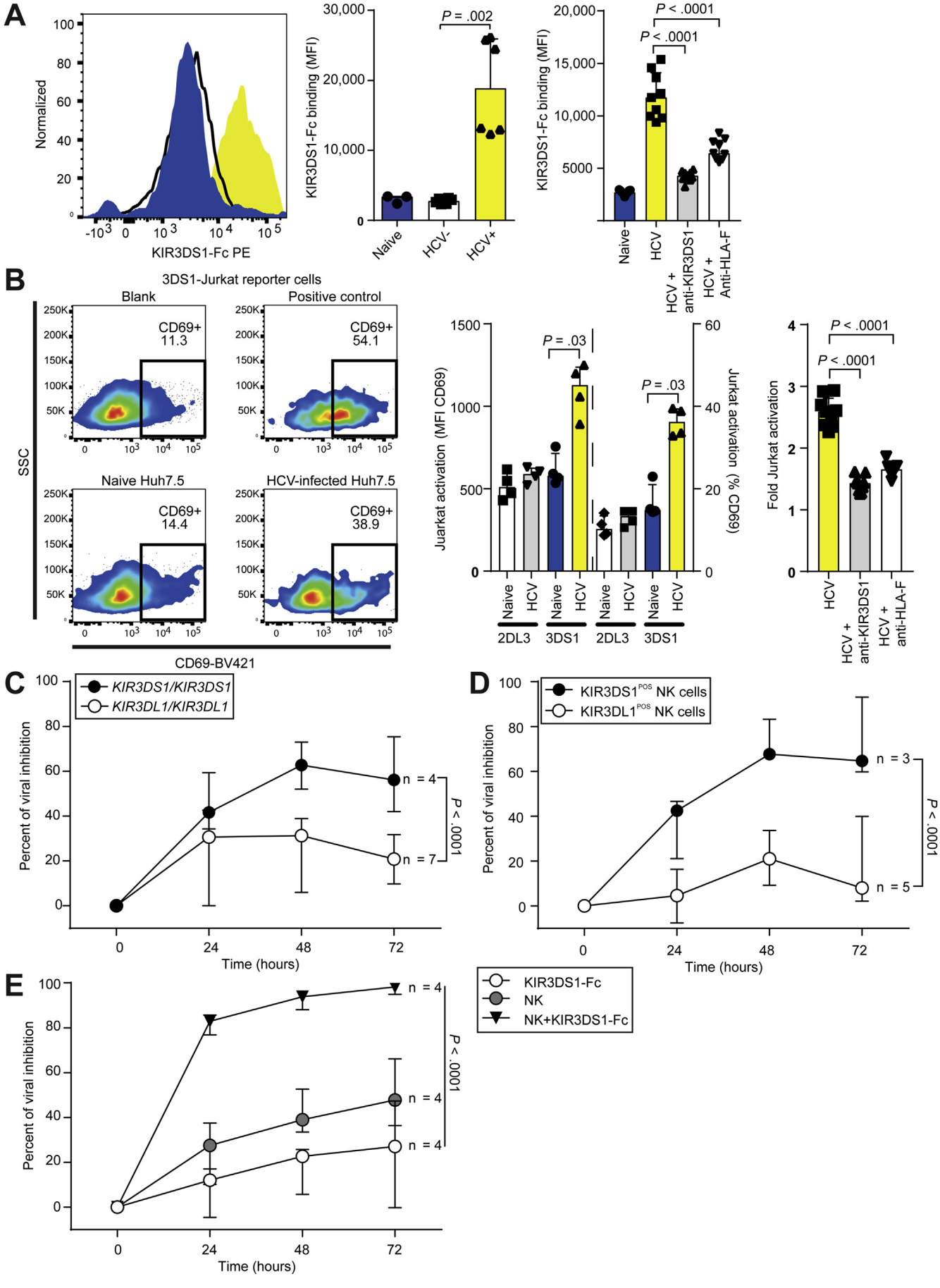
To determine whether HCV infection can cause an up-regulation of HLA-F, we used cell culture systems, chimeric humanized mouse models, and primary liver tissue samples from HCV-infected individuals (Supplementary Methods). Huh7.5 cells infected with an HCV Jc1-eGFP reporter virus exhibited significantly higher surface expression of HLA-F compared to HCV^{neg} Huh7.5 cells, which showed no expression of HLA-F (Figure 1*A* and *B*; Supplementary Figure 1). To assess whether HCV infection also induced HLA-F expression in vivo, livers of uPA/SCID/beige mice were repopulated with primary human hepatocytes and infected with HCV.[6] Expression of HLA-F occurred only in livers of HCV-infected animals, which also exhibited a significant increase of HLA-F messenger RNA levels (Figure 1*C*). In comparison to HLA-F expression on Huh7.5 cells, a more broadly distributed increase of HLA-F expression was observed in liver tissues of HCV-infected chimeric mice. HCV-induced cytokine production and enhancement of innate immune genes might have contributed to this broader expression of HLA-F within livers of infected mice. In contrast, intrinsic innate responses of Huh7.5 hepatoma cells are impaired, as Huh7.5 cells are unable to produce type I interferon in response to viral infections due to a dominant negative mutation in the RIG-I gene.[7] Finally, liver biopsy tissues from HCV-infected individuals exhibited clusters of cells with high HLA-F expression. Comparison with healthy liver tissues, derived from patients undergoing tumor resection surgery, revealed that areas of increased HLA-F expression were significantly more frequent in liver biopsies of HCV-infected individuals (Figure 1*D*). Taken together, these data using different model systems and primary liver samples demonstrate significant HLA-F induction following HCV infection.

To address the functional relevance of the observed up-regulation of HLA-F on HCV-infected cells, a soluble chimera of the extracellular domain of KIR3DS1 fused to the Fc region of human immunoglobulin G1 (KIR3DS1-Fc) was used (Supplementary Methods). Significantly increased binding of KIR3DS1-Fc to HCV^{pos} compared with HCV^{neg} or naïve Huh7.5 cells was observed, and KIR3DS1-Fc-binding was blocked by antibodies against HLA-F (Figure 2*A*). Furthermore, co-cultures of KIR3DS1ζ-Jurkat reporter cells with HCV-infected cells resulted in a significantly increased activation compared with co-cultures with uninfected Huh7.5 cells, which was abrogated through antibodies blocking KIR3DS1 or HLA-F (Figure 2*B*). These results demonstrate that HLA-F expression on HCV-infected cells leads to significant binding of KIR3DS1 and activation of KIR3DS1-expressing reporter cells. The stringent control of HLA-F expression[8] and its interaction with the activating NK cell receptor KIR3DS1 share homology with other pathways involving activating NK cell receptors, such as activation of $NKG2D^{+}$ NK cells by stress ligands like MIC-A and MIC-B, which are also up-regulated during infection.[9] This similarity is further reflected by the very limited sequence diversity of both KIR3DS1[10] and HLA-F, indicating a critical evolutionary role, in contrast to its inhibitory counterpart KIR3DL1 and its HLA-B ligands, which are highly diversified.[10]

Figure 1. HCV infection increases HLA-F expression. (*A*) HLA-F surface expression (MFI) on naïve (blue), HCV^{neg} (white/black line) and HCV^{pos} (yellow) Huh7.5 cells (exemplary histograms and bar graphs). Fold increase of HLA-F (dark gray), HLA-ABC (light gray), and HLA-E (white) expression on HCV-infected Huh7.5 cells (right). (*B*) Left to right, Jc1-infected Huh7.5 cells stained for Hoechst, HLA-F, or expressing a MAVS-NLS-RFP used to identify HCV-infected cells by nuclear translocation, respective overlay and magnification of white quadrant area. Scale bar is set to 10 μm. Quantification of $HLA\text{-}F^{pos}$ cells in 25 independent pictures (right). (*C*) Representative liver section from 1 uninfected and 1 HCV-infected humanized chimeric mouse, respectively, stained with Nup62 (red), 4′,6-diamidino-2-phenylindole (DAPI) (blue), and HLA-F (green). Quantitative reverse transcription polymerase chain reaction of RNA derived from liver tissues of uninfected control animals (blue, $n = 10$) and HCV-infected (yellow, $n = 5$) humanized chimeric mice. (*D*) Representative liver biopsy sections from one HCV^{neg} control individual and one HCV^{pos} individual, respectively, stained for HLA-F. The lower row depicts the threshold setting to identify HLA-F spots. Right: quantification of HLA-F spots per cell in HCV^{pos} individuals (yellow) and HCV^{neg} controls (white) Spots were quantified in 47 independent pictures from 6 different HCV^{pos} and 30 independent pictures from 6 different HCV^{neg} individuals. Medians with interquartile ranges are shown in (*A*), whereas box and whiskers are used in (*B*), (*C*), and (*D*). For the analysis of assays using replicates (*D*) linear mixed effect regression models were used to take into account the intraparticipant correlation

Given the expression of HLA-F on HCV-infected cells, the role of KIR3DS1/HLA-F interactions in controlling in vitro HCV replication was investigated. Huh7 cells were infected with an HCV Jc1 reporter virus expressing gaussia luciferase and co-cultured with primary human NK cells to longitudinally assess HCV replication (Supplementary Methods). NK cells were derived from healthy donors that were either *KIR3DS1* homozygous or *KIR3DL1* homozygous.




A
Normalized
KIR3DS1-Fc PE
KIR3DS1-Fc binding (MFI)
P = .002
Naive
HCV-
HCV+
P < .0001
P < .0001
Naive
HCV
HCV + anti-KIR3DS1
HCV + Anti-HLA-F
B
3DS1-Jurkat reporter cells
Blank
Positive control
Naive Huh7.5
HCV-infected Huh7.5
CD69+ 11.3
CD69+ 54.1
CD69+ 14.4
CD69+ 38.9
SSC
CD69-BV421
Juarkat activation (MFI CD69)
Jurkat activation (% CD69)
P = .03
P = .03
Naive
HCV
2DL3
3DS1
Fold Jurkat activation
P < .0001
P < .0001
HCV
HCV + anti-KIR3DS1
HCV + anti-HLA-F
C
KIR3DS1/KIR3DS1
KIR3DL1/KIR3DL1
Percent of viral inhibition
Time (hours)
n = 4
n = 7
P > .0001
D
KIR3DS1POS NK cells
KIR3DL1POS NK cells
Percent of viral inhibition
Time (hours)
n = 3
n = 5
P > .0001
E
KIR3DS1-Fc
NK
NK+KIR3DS1-Fc
Percent of viral inhibition
Time (hours)
n = 4
n = 4
n = 4
P > .0001

Independent of the genetic background, NK cells from all donors suppressed HCV replication in vitro. NK cells from *KIR3DS1* homozygous donors, however, exhibited a significantly higher antiviral capacity compared with NK cells from *KIR3DL1* homozygous individuals (Figure 2*C*). Of note, Huh7 and Huh7.5 cells are homozygous for the *HLA-B*54:01* (HLA-Bw6) allele,[11] ensuring that reduced inhibition of HCV replication was not due to inhibition of KIR3DL1^{pos} NK cell function by HLA-Bw4. To further elucidate a direct effect of KIR3DS1 in NK cell–mediated inhibition of HCV replication, KIR3DS1^{pos} and KIR3DS1^{neg} NK cells from a *KIR3DS1/KIR3DL1* heterozygous donor were sorted and compared in their antiviral activity, demonstrating a significantly stronger inhibition of HCV replication by KIR3DS1^{pos} vs KIR3DS1^{neg} NK cells derived from the same donor (Figure 2*D*). The observation of high antiviral capacity of KIR3DS1-expressing NK cells is in line with previous findings in HIV-1 infection,[5] and provides a mechanism for the reported associations between KIR3DS1 with viral clearance in HCV infection.[2,3] As most KIR3DS1+ individuals (>90%) also encode for KIR3DL1, the described protective effect of KIR3DS1 in conjunction with HLA-B molecules that allow binding to KIR3DL1[2,3,10] might reflect the combined effect of KIR3DL1-mediated licensing of NK cells and KIR3DS1-mediated activation of these licensed NK cells in response to HLA-F expression.

Therapeutic antibodies directed against NK cell receptors or their cellular ligands have been shown to be powerful modulators of antitumor and antiviral NK cell immunity in vivo, and antibodies blocking inhibitory KIRs are used in phase I/II clinical trials as immunotherapies for patients with lymphoma.[12,13] Although these studies aim to block inhibitory signals to unleash NK cell activity, KIR-Fc constructs have the potential to trigger specific activating signals via binding to CD16/FcγRIII expressed on NK cells. KIR3DS1 is expressed in approximately 40% (ranging from 80% to just 0.7%) of humans,[14] and KIR3DS1-Fc constructs can overcome this genetic limitation by enabling all individuals to mount CD16^{pos} NK cell–mediated cytotoxic responses against HLA-F–expressing target cells. Indeed, addition of KIR3DS1-Fc constructs to NK cells from *KIR3DS1*-negative donors in an HCV viral replication inhibition assay led to a significant reduction of viral replication (Figure 2*E*). As KIR3DS1 has been linked to reduced development of hepatocellular carcinoma in patients with HCV,[4] KIR3DS1-Fc fusion constructs might also represent a novel immunotherapeutic tool for antitumor therapies.[15] Taken together, the present study provides new insights into the molecular mechanisms mediating the protective effect of KIR3DS1 in HCV infection, and rational to harness the interaction between KIR3DS1 and HLA-F for therapeutic approaches in infectious diseases and cancer.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at https://doi.org/10.1053/j.gastro.2018.07.019.

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Figure 2. KIR3DS1^{pos} NK cells are activated by HCV-infected cells and control HCV replication. (*A*) Representative histograms of KIR3DS1-Fc binding to naïve (blue), HCV^{neg} (white), and HCV^{pos} (yellow) Huh7.5 cells (*left*). KIR3DS1-Fc binding to naïve (blue) and HCV^{pos} (yellow) Huh7.5 cells (middle, n = 3–6) in the presence and absence of blocking antibodies (right, n = 3–9). (*B*) Activation of KIR3DS1ζ- and KIR2DL3ζ-Jurkat reporter cells cultured alone (blank), with anti-KIR3DS1/3DL1–coated beads (positive control), naïve, or HCV-infected Huh7.5 cells (representative plots are shown in the left panel, middle panel shows quantification over n = 4 experiments), or in the presence and absence of blocking antibodies (right, n = 9). (*C*) Inhibition of HCV replication in Huh7 cells co-cultured with NK cells from *KIR3DS1* homozygous (black; n = 4) and *KIR3DL1* homozygous (white; n = 7) donors; (*D*) KIR3DS1^{pos} (black) (n = 3) and KIR3DL1^{pos} (white) (n = 5) sorted NK cells from *KIR3DS1/KIR3DL1* heterozygous individuals (n = 3–5) and (*E*) with NK cells from *KIR3DL1* homozygous (n = 4) donors with or without KIR3DS1-Fc constructs. Median with interquartile range is shown. For the analysis of assays using replicates (*C*) and/or repeated measurements over time (*C–E*), linear mixed effect regression models were used to take into account the intraparticipant correlation.

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Author names in bold designate shared co-first authorship.

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Reprint requests
Address requests for reprints to: Marcus Altfeld, MD, Department of Virus Immunology, Heinrich Pette Institute, Leibniz Institute for Experimental Virology, Martinistraße 52, 20251 Hamburg, Germany. e-mail: marcus.altfeld@leibniz-hpi.de; fax: 49-0-40-58051-103.

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Author contributions: SL and MA designed the study and wrote the paper. SL, AS, and JK performed experiments and analyzed the data. PF performed experiments. AH, WGB, and CK generated reporter cell lines and helped with assay design and establishment. AL, LH, TP, GM, AZ, JSzW, CS, and KO enrolled study participants, and provided and processed patient material. LR performed statistical analysis. EH provided the HCV cell culture systems. MD provided the chimeric mouse system. All authors provided continuous critical review of the data and commented on the manuscript.

Conflicts of interest
The authors disclose no conflicts.

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Supplementary Methods

Study Participants and Samples

HCV^{pos} individuals undergoing liver biopsies at the I. Department of Medicine, Section Infectious Diseases, University Medical Center Hamburg-Eppendorf, and HCV^{neg} individuals undergoing liver surgery due to tumor metastasis at the Department of General and Abdominal Surgery, Asklepios Hospital Barmbek, were included in this study. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors by standard density centrifugation. The study was approved by the ethics committee of the Ärztekammer Hamburg, and informed consent was obtained from all study participants and all experiments were performed in accordance with the relevant guidelines and regulations. Participant data for healthy donors and liver specimens are shown in the Supplementary Tables 1 and 2.

Animals

Homozygous uPA/SCID/beige mice were housed and maintained under specific pathogen-free conditions in accordance with the European Communities Council Directive (86/EEC) and protocols approved by the Ethical Committee of the city and state of Hamburg, as previously reported.[6]

Cell Culture and In Vitro HCV Infection

Huh7 cells kindly provided by R. Bartenschlager and Huh7.5 cells kindly provided by C.M. Rice were grown under standard cell culture conditions. For HCV infection, cell culture–derived viral stocks were used. For this culture, supernatant of Huh7.5 cells transfected with HCV RNA was harvested, filtered, and concentrated by ultrafiltration using Amicon Ultra-15 100 kDa filtration devices (Millipore, Bedford, MA) or left unconcentrated. For infection experiments, cells were seeded to tissue culture flasks and infected with the respective HCV strain 6 to 24 hours post-seeding.

Isolation of Primary Human NK Cells

For the co-culture experiments, primary human NK cells were either negatively enriched from isolated PBMCs of *KIR3DS1* homozygous and *KIR3DS1/3DL1* heterozygous healthy donors, using the EasySep Human NK Cell Enrichment Kit by Stemcell Technologies (Vancouver, Canada), or sorted from *KIR3DS1/KIR3DL1* heterozygous donors using a BD FACSAria Fusion instrument (BD Biosciences, San Jose, CA) and antibodies to identify KIR3DS1+ (z27.3.7) and KIR3DL1+ (DX9) single positive NK cells.

KIR Binding Assay

KIR binding assays were performed as previously described.[5] In short, KIR binding was analyzed on HCV+, HCV−, and naïve Huh7.5 cells by staining with KIR3DS1–immunoglobulin G fusion constructs (R&D Systems, Minneapolis, MN) for 1 hour on ice, secondary staining with goat anti-human immunoglobulin G(Fc) $F(ab')_2$ (Thermo Fisher Scientific, Waltham, MA) was performed for 30 minutes on ice. Afterward, cells were fixed (4% paraformaldehyde in phosphate-buffered saline) and analyzed using flow cytometry. Anti-KIR3DS1/3DL1 (Z27.3.7; Beckmann Coulter, Brea, CA) and anti-HLA-F (3D11; BioLegend, San Diego, CA) were used at a final concentration of 25 μg/mL to block the interaction of the KIR31DS1-Fc with their infected target cells. All antibodies used are listed in Supplementary Table 3.

$KIR\zeta^+$ Jurkat Reporter Cell Assay

$KIR\zeta^+$ Jurkat reporter cells were incubated with target cells at a reporter/target cell ratio of 1:10 at 37°C/5% CO_2 for 5 hours. Beads coated with anti-KIR3DS1/3DL1 (anti-KIR3DS1-L1, REA168; Miltenyi, Bergisch Gladbach, Germany) or uncoated were used as negative or positive controls, respectively. After coincubation, cells were stained with anti-human CD3-PerCP/Cy5.5 (UCTH1; BioLegend) and anti-human CD69-BV421 (FN50; BioLegend), followed by fixation and sample acquisition. Frequency of $CD69^+$ reporter cells as well as surface expression of CD69 was assessed by flow cytometry and used as a measure of reporter cell activity. Anti-KIR3DS1/3DL1 (Z27.3.7; Beckmann Coulter) and anti-HLA-F (3D11; BioLegend) were used at a final concentration of 25 μg/mL to block the interaction of the reporter cells with their infected target cells. All antibodies used are listed in Supplementary Table 3.

Infection and Treatment of Chimeric Mice

Human liver chimeric mice were generated as previously reported.[6] To establish HCV infection, animals received a single intraperitoneal injection of a patient-derived HCV inoculum (1×10^7 HCV-RNA copies/mL, genotype 1a). Viral loads were monitored 2 to 4 weeks postinfection as previously described.[6]

Reverse Transcriptase–Polymerase Chain Reaction

To determine HLA-F gene expression levels, human-specific primers (Hs04185703_gH; Life Technologies, Carlsbad, CA) from the TaqMan Gene Expression Assay System were used and samples were analyzed in the ViiATM 7 Real-Time PCR System (Life Technologies). The mean of the human housekeeping genes GAPDH and ribosomal protein L30 was used to normalize human gene expression levels.

Immunohistochemistry of Cell Lines

For immunofluorescence, Huh7.5 cells transduced with MAVS-RFP-NLS were infected with Jc1wt (multiplicity of infection 0.025), reseeded to coverslips, and fixed 5 days postinfection with 4% paraformaldehyde for 1 hour at 4°C. The MACS-RFP-NLS construct allows for identification of HCV-infected cells, as on infection and cleavage of mitochondrial antiviral-signaling protein (MAVS) the SV40 nuclear localization sequence (NLS) leads to nuclear translocation of the red fluorescence protein (RFP). After permeabilization with 0.1% Triton-X-100 for 5 minutes at room temperature and incubation in blocking solution (50 mM Tris, 5% bovine serum albumin, 1% fish skin gelatine, 0.15% glycine), cells were incubated with

anti-HLA-F (3D11; BioLegend) (1:100) overnight at 4°C followed by the secondary antibody donkey-anti-mouse-Alexa Fluor 488 (Thermo Fisher), 1:1500) for 1 hour at room temperature. Nuclei were stained with Hoechst. Samples were embedded in Mowiol (Applichem, St Louis, MO) and analyzed on a Nikon (Tokyo, Japan) C2plus confocal scanning microscope with an Apo x60 Oil λS DIC N2 oil objective. All antibodies used are listed in Supplementary Table 3.

Immunohistochemistry of Chimeric Mice Samples

Cryosections (12 μm) of mouse livers were stained with mouse monoclonal anti-human Calnexin (Cell Signaling, Leiden, the Netherlands) to identify human hepatocytes in mouse livers. Mouse monoclonal anti-HCVcore (Abcam, Cambridge, UK) was used to detect infected cells. For HLA-F detection, mouse livers were costained with rat monoclonal anti-human Nucleoporin p62 (Abcam) and mouse anti-human HLA-F (3D11; BioLegend). Slides were fixed in acetone for 10 minutes at room temperature and permeabilized with 0.1% Triton-X for 5 minutes followed by 1-hour TNB Block. In a secondary staining, specific signals were visualized with goat anti-rat 546-labeled secondary antibodies or TSA Fluorescein System (Perkin Elmer, Waltham, MA) by using anti-mouse horseradish peroxidase–labeled antibody and nuclear staining was achieved by DRAQ5 (1:2000 dilution; Axxora, Lörrach, Germany). Stained sections were mounted with fluorescein mounting media (Dako, Glostrup, Denmark) and analyzed by confocal laser scanning microscopy (Microscope Biorevo BZ-9000; Keyence, Osaka, Japan) using the same settings for the different experimental groups. All antibodies used are listed in Supplementary Table 3.

Immunohistochemistry of Human Liver Samples

Cryosections (8 μm) of human livers were stained with mouse anti-human HLA-F (3D11; BioLegend) and 4′,6-diamidino-2-phenylindole (DAPI) (Life Technologies, Darmstadt, Germany). Slides were fixed in acetone for 10 minutes at room temperature and permeabilized with 0.1% Triton-X for 5 minutes. In a secondary staining, specific signals were visualized with donkey-anti-mouse Alexa Fluor 555 (Thermo Fisher)–labeled secondary antibody. Stained sections were embedded in Mowiol (Mowiol 4-88; Applichem) and analyzed by confocal laser scanning microscopy (Nikon C2plus confocal scanning microscope with an Apo x60 Oil λS DIC N2 oil objective) using the same settings for the HCV^{pos} and HCV^{neg} samples. Spots were quantified in 47 independent pictures from 6 different HCV^{pos} and 30 independent pictures from 6 different HCV^{neg} individuals. All antibodies used are listed in Supplementary Table 3.

Viral Inhibition Assay

Inhibition of viral replication was measured using a Jc1 reporter strain carrying a gaussia luciferase between p7 and NS2. Luciferase activity was used as a measure for viral replication in this system. In short, Huh7 cells were infected with the Jc1 reporter virus. After 6 days, cells were reseeded to 24-wel -plates with 1.5 x 10^4 cells per well. Seven days after infection, cells were co-cultured at an effector to target ratio of 2:1 with isolated primary human NK cells for 3 days. Every 24 hours for 72 hours, 50 μL supernatant was removed and replaced with fresh medium. The gaussia luciferase activity in the supernatant was determined using the Renilla Luciferase Assay System (Promega, Madison, WI). Twenty microliters of supernatant was lysed for 1 hour at room temperature by adding the same volume of 2X Renilla Luciferase Assay Lysis buffer (Promega), and luciferase activity was measured on a luminometer with injector (Infinite M200 plate reader; Tecan Group Ltd, Männedorf, Switzerland). All measurements were performed in triplicate to reduce variability.

Flow Cytometry

For flow cytometry analysis of HLA-F up-regulation in HCV-infected and uninfected cells, Huh7.5 cells were infected with a Jc1-eGFP reporter virus. To support viral spread, cells were trypsinized and reseeded 1-day post infection. To determine expression of HLA-F, cells were stained with a monoclonal anti-human HLA-F-PE antibody (3D11; BioLegend) for 20 minutes at room temperature followed by fixation and data acquisition. All antibodies used are listed in Supplementary Table 3.

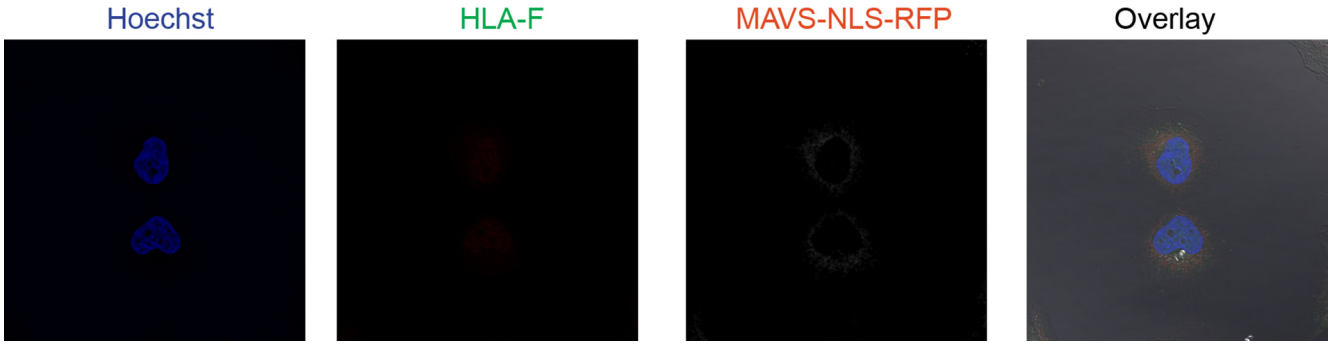
Data Acquisition, Analysis, and Statistics

Flow cytometry was performed on an LSRFortessa and FACSCanto II (BD Bioscience) and analyzed using FlowJo software v10 (Tree Star, Inc., Ashland, OR). Figures were designed and statistical analysis done using GraphPad Prism 7 (GraphPad Software, Inc., San Diego, CA) and SAS version 9.3 (SAS Institute, Cary, NC). In general, if not stated otherwise, bar graphs show the median and error bars indicate the interquartile range. Due to the sample size and because data were assumed to be of non-gaussian distribution, the nonparametric Mann-Whitney test was used to assess statistical differences between 2 groups. Comparisions between multiple groups were performed using Kruskal-Wallis test with Dunns corrections for multiple comparisons. If multiple groups are shown in one graph, bracketed lines indicate comparisons between 2 selected groups, whereas straight lines indicate comparison between all groups.

For the analysis of assays using replicates (Figures 1*D* and 2*C*) and/or repeated measurements over time (Figure 2*C*–*E*), linear mixed effect regression models were used to take into account the intraparticipant correlation.

Data Availability

The data that support the findings of this study are available from the corresponding author on reasonable request.



Supplementary Figure 1. HLA-F expression on naïve Huh7.5 cells. Naive Huh7.5 cells were stained for Hoechst, HLA-F, or expressing a MAVS-NLS-RFP.