Effect of HLA class I-peptides on KIR+ NK cell function in the context of viral infections

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This thesis is dedicated to the memory of my beloved grandmother Annette Chapel

Abstract

Background & Aims: NK cells play an important role in viral infection and distinct KIR/HLA associations have shown to influence viral disease outcome, in particular for HCV and HIV-1 infection. Viral infections are able to alter the repertoire of peptides presented by HLA class I molecules and thereby impact KIR/HLA interactions. We aim to identify HLA/peptide complexes binding to the inhibitory NK cell receptor KIR2DL3 and the activating NK cell receptor KIR2DS1 to gain a better understanding of how HLA class I-ligands modulate KIR binding and thus influence NK cell functions.

Methods: To investigate the influence of peptides presented by the HLA-C on KIR2DL3 and KIR2DS1 binding, we established reporter cell assays which measure the response of KIR2DL3-IgG fusion construct to peptide-pulsed 721.221.TAP1ICP47-HLA-C*03:04 cells and KIR2DS1-transfected Jurkat cells to peptide-pulsed 721.221.TAP1KO-HLA-C*06:02 cells. These assays were used to screen a large panel of HCV, HIV-1 virus-derived and synthetic peptides. Primary NK cells and NK cell clones were isolated from healthy donors to investigate how the peptides identified impacted KIR2DL3$^+$ and KIR2DS1$^+$ NK cell function.

Results: One HCV peptide "YIPLVGAPL" was identified to induce strong binding of KIR2DL3 to 721.221.HLA-C*03:04 cells and inhibited KIR2DL3$^+$ NK cell function. This sequence exhibited a high frequency of mutations in different HCV genotypes and point mutations on the peptide showed lower ability to inhibit KIR2DL3$^+$ NK cell function. On the contrary, one synthetic HLA-C*06:02-presented peptide, "SRGPVHHLL", was identified to induce strong binding of KIR2DS1 and increase the activation of primary KIR2DS1$^+$ NK cell clones. No peptide virus-derived was identified binding to KIR2DS1.

Conclusions: We successfully identified novel HLA/peptide complexes as ligands for KIR2DL3 and KIR2DS1 and confirmed that HLA class I peptides impact KIR$^+$ NK cell functions. Our results suggest that one HCV derived HLA class I peptide can impact KIR2DL3$^+$ NK cell functions and that naturally occurring sequence mutations in the peptide alter these interaction making the inhibition less efficient, providing potential pathways for viral escape. On the other hand, we confirmed the peptide-dependent binding of the activating NK cell receptor KIR2DS1 and our results suggested that an additional factor might be required to induce effective *in vivo* KIR2DS1$^+$ NK cell function.

Table of contents

List of scientific publications

This thesis is presented as cumulative and based on the following papers:

1. Sebastian Lunemann, Gloria Martrus, Angelique Hölzemer, **Anais Chapel**, Maja Ziegler, Christian Körner, Wilfredo Garcia Beltran, Mary Carrington, Heiner Wedemeyer, Marcus Altfeld

Sequence variations in HCV core-derived epitopes alter binding of KIR2DL3 to HLA-C∗03:04 and modulate NK cell function.

Journal of Hepatology 04/2016; DOI:10.1016/j.jhep.2016.03.016

2. **Anais Chapel**, Wilfredo F. Garcia-Beltran, Angelique Hölzemer, Maja Ziegler, Sebastian Lunemann, Gloria Martrus, Marcus Altfeld

Peptide-specific engagement of the activating NK cell receptor KIR2DS1.

Scientific Reports 05/2017; 7(1); DOI:10.1038/s41598-017-02449-x

Other scientific publication not included in this thesis:

3.Claudia Beisel, Susanne Ziegler, Glòria Martrus Zapater, **Anaïs Chapel**, Morgane Griesbeck, Heike Hildebrandt, Ansgar W. Lohse, Marcus Altfeld

TLR7-mediated activation of XBP1 correlates with the IFNα production in humans.
Cytokine 04/2017, DOI:10.1016/j.cyto.2017.04.006

List of abbreviations

AA: Amino Acid

ADCC: Antibody-dependent cell-mediated Cytotoxicity

AIDS: Acquired Immunodeficiency Syndrome

aKIR: activating KIR

ART: AntiRetroviral Treatment

CHO: Chinese Hamster Ovaries

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

DAA: Direct-Acting Antiviral

DC: Dendritic Cells

DNA: DeoxyriboNucleic Acid

DRiPs: Defective Ribosomal Products

EBV: Epstein–Barr virus

ER: Endoplasmic Reticulum

EVT: Extravillous Trophoblasts

GWAS: Genome-Wide Association Studies

HAART: Highly Active Antiretroviral Therapy

HCMV: Human CytoMegaloVirus

HCV: Hepatitis C Virus

HFFF: Homozygous Fetal Foreskin Fibroblasts

HIV-1: Human Immunodeficiency Virus type 1

HLA: Human Leucocyte Antigen

hNK cells: intrahepatic NK cells

HPCs: Hematopoietic Progenitor Cells

HSC: Hepatic Stellate Cells

HSV-1: Herpes Simplex Virus type 1

IFN: Interferon

iKIR: inhibitory KIR

IN: Integrase

ITAMs: Immunoreceptor Tyrosine-based Activation Motifs

ITIMs: Immunoreceptor Tyrosine-based Inhibitory Motifs

IUGR: Intra Uterine Growth Restriction

KIR: Killer-Immunoglobulin like Receptor

LCR: Leukocyte Receptor Complex

LTRs: Long Terminal Repeat sequences

MCMV: Murine CytoMegaloVirus

MHC class I: Major Histocompatibility Complex class I

MS: Mass Spectrometry

NCR: Natural Cytotoxicity Receptor

NK cells: Natural Killer cells

OC: Open Conformers

ORF: Open Reading Frame

PBMC: Peripheral Blood Mononuclear Cell

PLC: Peptide Loading Complex

pNK cells: peripheral blood NK cells

PR: Protease

PTMs: Post-translational modifications

RNA: RiboNucleic Acid

RT: Retrotranscriptase

SIV: Simian Immunodeficiency Virus

SNP: Single Nucleotide Polymorphism

TAP: Transporter associated with Antigen Processing

TCR: T-cell receptor

uNK cells: uterine NK cells

WHO: World Health Organization

WT: Wild Type

β2m: β2 microglobulin

INTRODUCTION

Hepatitis C Virus (HCV) and Human Immunodeficiency Virus type 1 (HIV-1) are responsible for major global viral epidemics, ranking as two of the most important public health problems worldwide[1]. Although differing in their virological properties and pathogenesis, HCV and HIV-1 both cause chronic infections with respectively 71 million and 40 million chronically infected individuals worldwide[1,2]. For HCV, a recent breakthrough in treatment with new drugs, the newly Direct-Acting Antiviral (DAA) therapies, has led to an effective cure of chronic HCV infection[3]. The treatment for HIV-1, the Highly Active Antiretroviral Therapy (HAART), allows for an efficient and stable viral control in infected individuals, improving their life expectancy and life quality. However, these life-long therapies remain costly and are not always available for infected individuals in developing countries. Ultimately, the development of preventive vaccines remains the most effective way to reduce HCV and HIV-1 infection rates. Vaccine efforts have been mainly focused on harnessing the adaptive immune response, however, these attempts have not been successful so far[4,5]. On the contrary, a growing number of studies highlight the role of the innate immune system to determine the clinical outcome of HCV and HIV-1. Indeed, the innate immune response plays an essential role in containing the virus within the first hours following infection and takes part in shaping the adaptive immune response[6], determining the quality of the adaptive HCV/HIV-1 specific response[7]. As the main cellular effector of the innate immune system, studies of Natural Killer (NK) cells are of particular interest in order to better understand which factors allow the control or, on the contrary, the persistence of viral infections. In addition, host genetic factors linked to genes coding for NK cell receptors have been associated with improved disease outcome in HCV as well as HIV-1[8,9]. Therefore, studying innate immune responses and particularly NK cell response to HIV-1 and HCV remains important to gain new insights into the interplay between host immune response and establishment of a persistent viral infections, and might help uncover new strategies to develop preventive (or therapeutic) immunotherapies.

I. HCV

a. Molecular biology and course of infection

Hepatitis C Viruses (HCV) belong to the Flaviviridae family. During the acute phase of infection HCV often only cause mild symptoms such as decreased appetite, fatigue, nausea, pain in muscles and joints, and weight loss. Whilst it can resolve spontaneously (in about 20% of cases)[10], HCV infection leads to chronic hepatitis in the majority of infected individuals. Individuals infected with chronic HCV infection develop progressive liver fibrosis, resulting frequently in liver cirrhosis, with high risks of developing hepatocellular carcinoma[3]. As a consequence, chronic HCV infection remains one of the leading causes for liver transplantation in the Western World[1]. HCV infection is a blood borne virus and can be transmitted by contaminated blood products, poorly sterilized medical equipment, or intravenous drugs use. HCV can also be transmitted sexually and vertically from mothers to baby; however, these modes of transmission are much less common[11]. In 2016, approximately 71 million people were infected with chronic HCV worldwide[1]. Recent advances in treatment using newly developed DAA therapies improved the previous existing Pegilated-Interferon-γ (IFN-γ) and Ribavirin therapies. DAAs have been shown to effectively cure chronic HCV infections resulting in sustained virological responses rates of over 90%[5]. This effective HCV treatment has been achieved with a combination of drugs adapted to the HCV genotypes and stage of liver disease[3]. Nevertheless, this therapy remains costly and is unavailable to the majority of individuals worldwide. This is further complicated by the fact that only 20% of infected individuals worldwide have been diagnosed and of those only 7.4% were started on therapy in 2015[12]. As such, HCV remains a major global health issue.

b. Cellular targets

Human hepatocytes are the primary target cells of HCV *in vivo*[13]. As HCV infection of hepatocytes is considered non-cytopathic, the ensuing liver damage seems to be mainly immune mediated. The first response to HCV infection is thought to be IFN-β production by infected hepatocytes[14], which induces local antiviral defenses in the infected cells and liver tissues. IFN cytokines are antiviral cytokines and essential to recruit and modulate the actions of immune cells of the adaptive immune response[15]. Additionally, the development of robust HCV-specific $CD4^+$ T and $CD8^+$ T cells has been correlated with the spontaneous resolution of the infection[16]. HCV-specific T cells can suppress viral

replication and eliminate HCV-infected hepatocytes by intrahepatic production of IFN-γ[17] and direct cytolytic activity[18]. However, only a minority of individuals (around 20%) are able to spontaneously clear acute HCV acute infection while the majority progress to persistent viremia. It is likely that HCV has developed a number of mechanisms to evade host defenses, including high genomic variability that allows the alteration of its antigenic epitopes to escape immune surveillance[19,20] and suppression of host immune responses by HCV proteins[21].

c. Genetic organization

HCV genome consists of a positive-stranded RiboNucleic Acid (RNA) ~9 kb molecule (9024 to 9111 nucleotides depending on the genotype[22]) which encodes for a polyprotein precursor of approximatively 3000 Amino Acids (AA). This polyprotein is cleaved *co*- and *post*-translationally into three structural proteins (core, E1, E2) and seven nonstructural proteins (NS1, NS2, NS4A, NS4B, NS5A, NS5B)[23] *(Fig. I.1)*. Several of the viral proteins are involved in immune evasion, mainly by interfering with the IFN-α/β signaling pathways[24] (NS3-NS4A[25–27], NS4B[28], NS5A/E2[29] and the core protein[30]). HCV is characterized by its high genomic variability in its nucleotide sequence due to the high error-prone rate RNA-dependent RNA polymerase ($2.5x10^{-5}$ mutations per cycle per genome)[31], leading to the distinction of seven genotypes (1-7), and more than 100 subtypes within these. HCV genomic variability is not evenly distributed along its genome; it is mainly focused on the membrane glycoproteins E1 and E2[32]. This high genomic variability and evolvability confers HCV the ability to adapt to the host immune system and to even start developing resistances to new drugs treatments[33]. The prevalence of HCV genotypes varies geographically with HCV genotype 1 causing the majority of infections in Europe[3].

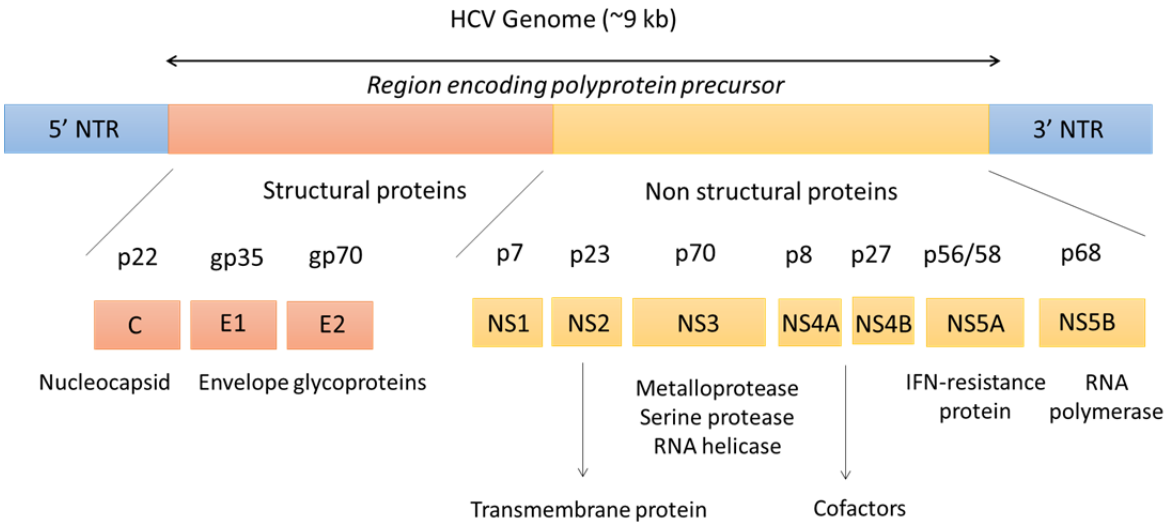

Fig I.1. HCV genome structure[22].

HCV genome code for a polyprotein precursor spliced post-translationally into three structural proteins (C, E1, E2) (orange) and seven nonstructural proteins (NS1, NS2, NS3, NS4A, NS4B, NS5A, NS5B) (yellow). Open Reading Frames (ORFs) are shown as rectangles.

II. HIV-1

a. Molecular biology and course of infection

HIV-1 is a human-specific lentivirus, which causes progressive failure of the immune system and ultimately results in the Acquired ImmunoDeficiency Syndrome (AIDS), a syndrome characterized by the occurrence of severe opportunistic infections and/or development of AIDS-related cancers such as Kaposi-Sarcoma or non-Hodgkins lymphoma[34]. HIV-1 can be detected in several body fluids such as blood, semen, vaginal fluid, pro-ejaculate and breast milk and, therefore, transmission mainly occurs sexually, through intravenous drug usage or vertically from mother-to-child[35]. In 2015, according to the World Health Organization (WHO), 36.7 million people were estimated to be infected with HIV-1 worldwide leading to 1.1 million AIDS related deaths[2]. These data demonstrate that HIV-1 still remains a global health issue. HAART treatment for HIV-1 consists of a combination of drugs targeting central steps in the HIV-1 life cycle such as reverse transcription, integration and protease processing, leading to suppressed viral replication to undetectable levels[36]. HAART has improved life expectancy and quality of HIV-1-infected individuals dramatically, but the need for lifelong treatment remains. Due to economic reasons, last-generation HAART is not accessible to the entire HIV-1-infected population. Additionally, HAART induces important clinical side effects such as

metabolic disorders, liver and kidney dysfunctions[37]. Another limitation of HAART is the development of drug resistances due to the high mutation rate of HIV-1. This is of growing concern because the number of HIV-1-infected individuals on HAART increases exponentially [38] and treatment interruptions due to non-adherence to HAART or limited access to HAART can lead to the development of multi-drug resistant HIV-1 strains. Consequently, there is an ongoing need to explore novel therapeutic approaches and for this purpose, revealing new insights into the cellular and immune mechanisms involved in the HIV-1 pathogenesis may help find new approaches to treat HIV-1.

b. Cellular targets

The main cellular target of HIV-1 are $CD4^+$ T cells, macrophages and Dendritic Cells (DC) [39], which are important cells of the human immune system, as well as astrocytes during primary HIV-1 infection[40]. HIV-1 infection of $CD4^+$ T cells leads to apoptosis through different mechanisms such as direct cell killing[41], apoptosis induced by viral proteins like env, tat, nef, vpu, vpr[42], cell death due to excessive activation-induced-cell-death of immune cells[43] and bystander apoptosis of neighboring uninfected cells[44]. $CD4^+$ T cells are also targeted and eliminated through killing by $CD8^+$ cytotoxic T cells[35] and NK cells[45]. When $CD4^+$ T cell numbers decline below a critical level, cell-mediated immunity is compromised, and the body becomes progressively more susceptible to opportunistic infections[46]. Moreover, HIV-1 persists in a minority of resting T cells where the DeoxyriboNucleic Acid (DNA) of the virus is integrated in the cell host genome, but is transcriptionally silent[47]. These latently infected cells are long-lived and constitute HIV-1 reservoirs which cannot be targeted by current AntiRetroviral Treatments (ARTs), thereby, representing a major obstacle to HIV-1 eradication [48].

c. Genetic organization

Phylogenetically, HIV is divided in two major types based on phylogenetic analysis: HIV-1 and HIV-2. HIV-1, in particular subtype M, is responsible for the majority of the observed human infections due to its higher virulence and infectiveness rates. There are at least nine genetically distinct clades of HIV-1 within the group M named clades A, B, C, D, F, G, H, J and K[49]. The dominant HIV-1 subtype in Americas, Western Europe, Australia and Japan is the clade B[50] and is the consequently the most studied subtype in HIV-1 clinical research.

HIV-1 genome consists of nine genes, flanked by two Long Terminal Repeat sequences (LTRs) *(Fig I.2)*. Three genes, *gag*, *pol* and *env,* encode for the major structural proteins and the essential enzymes[51]. *Gag* is highly conserved and encodes for the matrix (p17), capsid (p24) and nucleocapsid (p9). *Pol* encodes for the viral enzymes necessary for the virus replication cycle including the Reverse Transcriptase (RT), protease (PR) and integrase (IN). *Env* is genetically highly variable and encodes for the glycoproteins of the viral membrane including gp120 and gp41. In addition, HIV-1 contains six accessory genes (*vif, vpu, vpr, tat, rev* and *nef*). *Tat* and *rev* encode for essential viral regulatory proteins, which modulate transcriptional and post-translational steps of virus gene expression and are necessary for viral propagation[52]. The function of the genes *vif*, *vpu*, *vpr* and *nef* encoding for accessory proteins is not completely elucidated. However, *vif* promotes infectivity of the viral particle. *Vif* interacts also with the cellular APOBEC-3G protein, involved in innate antiretroviral immune activity against retroviruses by interfering with proper replication to trigger the ubiquitination and degradation[53]. *Vpu* has been shown to have two different roles: degradation of CD4 in the Endoplasmic Reticulum (ER) and extracellular release of the viral particles. *Vpr* is involved in targeting nuclear import of the preintegration complex, cell growth arrest, inhibition of cellular differentiation and transactivation of cellular genes. *Nef* allows for downregulation of CD4 and Human Leucocyte Antigen (HLA) class I molecules at the surface of the infected cell[52].

HIV-1 is characterized by a vast genetic diversity, even within one individual, resulting from a high level of mutation ($(4.1 \pm 1.7) \times 10^{-3}$ per base per cell))[54]. This extreme degree of diversity allows the virus to rapidly select for mutants escaping different arms of the immune system including escape to neutralizing antibodies and T cell responses, to develop drug resistance, and to evade new vaccination strategies.[55].

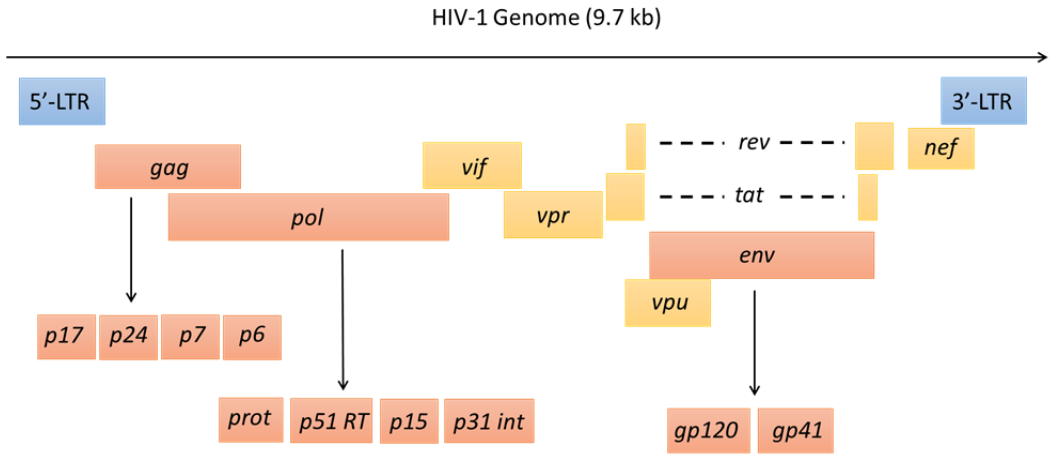

Fig I.2. HIV-1 genome structure[56].

The HIV-1 genome consists of three major genes (gag, pol and env) (orange) and six additional genes (vif, vpu, vpr, tat, rev and nef) (yellow) flanked by two LTRs (5′-LTR and 3′-LTR) (blue). ORFs are shown as rectangles.

III. Role of Natural Killer cells in HCV/HIV-1

The immune system can be defined as a set of mechanisms protecting the host against infections and malignancies. Its fundamental ability to distinguish between "non-self" and "self" molecules allows the immune system to recognize and specifically target a wide variety of pathogens such as viruses, bacteria and parasites[6]. In humans, the main effectors are leukocyte cells, which derive from stem cells within the red bone marrow. The human immune system is generally divided into two major categories: innate and adaptive. The innate immunity is the first unspecific response towards pathogens, while the adaptive immunity develops subsequently and responds precisely to a given antigen. Specifically, the innate immune response is fast but lacks the ability to generate immunity memory and to provide specific protective immunity that prevents reinfection[6]. If the infection persists, a slower but highly specific response, the adaptive immune response, overtakes and generates long-lived memory cells[57]. NK cells have been described as the main cellular effectors of the innate immune system and are able to kill and recognize abnormal and infected cells without prior sensitization[58] but share also some features of the adaptive immunity[59]. Indeed, studies in animal models such as mice and rhesus macaques highlighted that NK cells can acquire immunological memory features against haptens[60,61] or viral infections[62] which blur the distinction between the properties of NK cells and adaptive lymphocytes. Moreover, NK cells are able to efficiently contain viral infection in early stages and to shape the subsequent adaptive immune response[45] mainly

through interaction with DCs[63] and the production of immunoregulatory cytokines such as IFN-γ and TNF-α[64] and are, therefore, key players of the immune defense against viral infection including HCV and HIV-1.

a. Phenotype

In humans, NK cells originate from $CD34^+$ Hematopoietic Progenitor Cells (HPCs) residing in the bone marrow[65]. Generally, NK cells represent 5-10% of the lymphocytic population in the blood and approximately 30% of intrahepatic lymphocytes in healthy adults[66]. NK cells are defined as $CD14^-CD19^-CD3^-CD16^{+/-}CD56^+$ lymphocytes and are subdivided into two major categories based on CD16 (Fc_γRIII receptor) and CD56 cell surface expression[67,68]. Peripheral blood NK cells contain a majority of $CD16^+CD56^{dim}$ NK cells (90%) that express Killer-Immunoglobulin like Receptors (KIRs), while the rest are $CD16^{dim/-}CD56^{bright}$ NK cells (10%) with a lower expression of KIRs *(Fig.I.3)*. Both subsets are functionally distinct: $CD16^+CD56^{dim}$ NK cells have little proliferative capacity, produce moderate amount of cytokines but have a strong cytotoxicity activity due to the high presence of cytolytic granules, perforin and granzymes[69]. Contrarily, $CD16^{dim/-}CD56^{bright}$ NK cells proliferate and secrete a large amount of cytokines, including IFN-γ, TNF-α, IL-10, IL-13 and GM-CSF[70]. Of note, both subsets are generally described as different stages of NK cell maturation, progressing from $CD56^{bright}$ to $CD56^{dim}$ NK cells phenotype in peripheral blood [71,72]. Finally, a $CD3^-CD56^-CD16^-$ NK cell subset has also been described to expand in chronic viral infection such as HCV and HIV-1 and to represent an exhausted/anergic subset of NK cells[73].

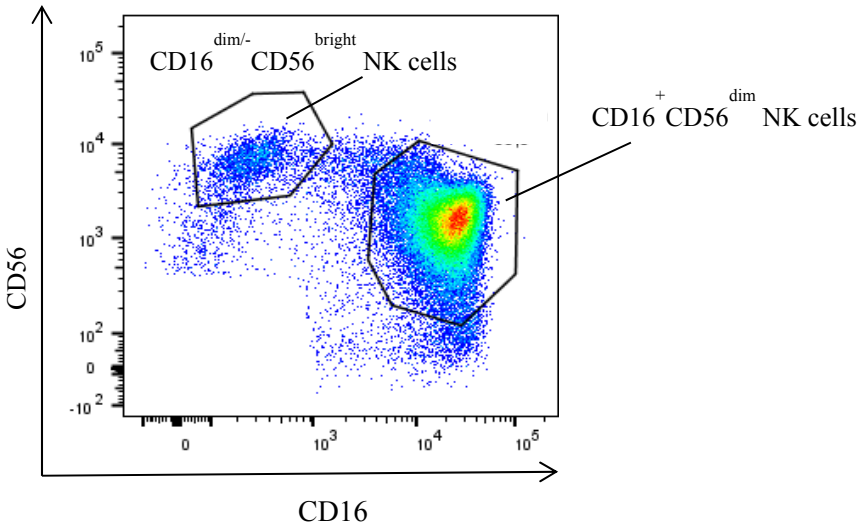

Fig I.3. Characterization of the two major NK cells subsets by flow cytometry.
NK cell populations are divided based on the relative density of CD16 and CD56 expression at the cell surface. $CD16^{dim/-}CD56^{bright}$ NK cells have a regulatory role while the $CD16^{+}CD56^{dim}$ have cytotoxic functions.

b. Effector functions

NK cells are recruited and activated by inflammatory cytokines and chemokines such as IFN-α, and their main effector function is the cytolysis of tumor or virus-infected cells in a cell-contact dependent manner[6]. This is mediated through the exocytosis of cytoplasmic granules containing perforin and granzymes[74,75], Fas ligand mediated induction of apoptosis[76] or Antibody-Dependent Cell-mediated Cytotoxicity (ADCC)[77]. Furthermore, NK cells play an important immunoregulatory role by producing soluble factors such as cytokines and chemokines as well as establishing cross-talks with other immune cells including DCs[63]. NK cell function has been widely and strongly implicated in the clinical outcome in diverse areas of the immune response; such as in cancer, organ transplantation, autoimmunity, reproduction and infectious diseases[45,78–83]. In the context of viral chronic infections, a substantial amount of studies have highlighted the importance of NK cells as key players in the first line of defense against both HCV and HIV-1.

Several epidemiological studies identified an association between alleles coding for the NK cell receptor *KIR2DL3* and its ligand, *HLA-C1* and HCV viral clearance in intravenous drug users[84–86] highlighting the role of NK cells in HCV infection. Moreover, *in vitro* studies showed that the HCV protein (E2) inhibits signaling in NK cells

suggesting an HCV escape mechanism to specifically evade those defenses[87–89]. These results were not however confirmed[90] and another study suggest that the HCV E2 protein does not modulate NK cell function and without direct inhibition by HCV, NK cells might become activated by cytokines in acute HCV infection and contribute to infection outcome and disease pathogenesis. The differences observed between the obtained results in these controversial studies are, however, probably due to different model systems used. NK cells have also been shown to suppress HCV replicon expression in human hepatocytes in an IFN-γ-dependent manner[91]. Additionally, intrahepatic NK cells have been demonstrated to be important determinant of liver progression in mouse models through direct killing of activated Hepatic Stellate Cells (HSCs) and/or production of IFN-γ, which inhibits HSC activation[92,93]. *In vivo,* the role of NK cells in controlling HCV still remains unclear but phenotypic and functional changes in NK cells subsets have been observed in both acute and chronic HCV[45,94,95], underlying the important role of NK cells in HCV. In acute HCV infection, the majority of studies describe an activated NK cell phenotype with high levels of IFN-γ production and elevated degranulation potential in acute HCV infected individuals[96,97], but no distinct pattern of receptor expression could be observed. The major problem when studying the role of NK cells in acute HCV infection is to pinpoint the exact date of infection. Patients are often asymptomatic and the incubation period between infection and onset of clinical symptoms of hepatitis may vary considerably. This leads to some discrepancies in the reports on the phenotype and function of NK cells in acute HCV infection. In addition, the lack of a fully immunocompetent small animal model renders difficult the analysis of the precise mechanisms involved in acute HCV infection[98]. On the other hand, NK cells from individuals with chronic HCV infection are more amenable to study. Most of the studies describe a decrease of peripheral blood NK cells in both absolute number and percentage of total lymphocyte population in chronic HCV individuals compared to healthy individuals[99–101]. However, it is not clear whether the reduction in NK cell frequency may be a consequence of HCV infection or a predisposing factor to chronic HCV infection. A relative increase of circulating $CD56^{bright}$, but not $CD56^{dim}$ NK cells is also described in chronic HCV[100,102] as well as an increase of the anergic subset of NK cells, $CD56^{-}CD16^{+}$ [97]. Additionally, alterations of receptors expression on the surface of NK cells can be observed. In particular, an increased number of peripheral blood and intrahepatic NK cells are described to bear the inhibitory receptor CD94/NKG2A[103,104]. Most of the studies showed discrepancies concerning the NK cell receptors, but an overall

increase in activating receptors including NKG2C, NKp44 and NKp30 is also suggested [97,101,103–107]. The link between alterations in NK cell phenotype and their anti-HCV function remains unclear with some studies describing an impairment of natural cytotoxicity in chronic HCV[104,106,107], while others suggest that the cytoxicity function is not reduced[99]. Taken together, changes in the NK cells compartment can be observed during chronic HCV infection but their consequences need further investigations.

NK cells have also been shown to have a crucial role during HIV-1 infection. One of the first studies linking NK cells to HIV-1 outcome showed that individuals possessing the allele coding for a NK cell receptor, *KIR3DS1,* and the *HLA-B* allele containing a Bw4 motif and an isoleucine at position 80 (HLA-Bw4-80I), have a slower progression to AIDS[108]. The mechanisms underlying the precise role of NK cells impacting HIV-1 disease progression requires further investigation. However, *in vitro*, NK cells efficiently mediate viral inhibition of HIV-1-infected $CD4^+$ T-cells[109]. Additionally, NK cells isolated from HIV-1-infected individuals have been shown to be impaired in their ability to kill HIV-1 infected autologous cells, as well as tumor cell lines[110]. This is in line with several studies showing that, *in vivo*, NK cells phenotype and functions are impaired by the effect of HIV-1 viremia[9,110–117]. It has been demonstrated that, in acute HIV-1 infection, a rapid expansion of cytotoxic $CD56^{dim}$ NK cells is observed before the $CD8^+$T cell expansion[118]. Moreover, as infection progresses, redistribution of NK cells subsets can be observed toward less functional subsets with a depletion of the cytotoxic $CD56^{dim}$ NK cells and an increase of the anergic $CD56^-CD16^+$ NK cells subset. This $CD56^-CD16^+$ NK cell subset expresses significantly higher levels of inhibitory receptors[112,113,115] and lower levels of Natural Cytoxicity Receptors (NCR)[111,113] compared with $CD56^+$ NK cells and is associated with decrease in global NK cell cytolytic activity[113] and cytokines secretion[116,119]. In contrast, HIV-1-infected individuals whose viremia was suppressed to below detectable levels by HAART for 2 years or longer, showed considerable improvement in their NK cell subsets with expression of CD56 and NK cell receptors comparable to NK cells from healthy individuals[115]. Altogether, the impaired function of NK cells from HIV-1 infected individuals may be due to elevated frequencies of highly dysfunctional $CD56^-$ NK cell subsets and therefore, may contribute to disease progression.

These studies showed the importance of NK cells in the first line of defense against both HCV and HIV-1; however, their precise role is not fully understood. The expression of NK cells surface receptors can be modified by specific cellular ligands expressed on HCV and HIV-1 infected cells[9,120] and several studies have highlighted their significant influence on HCV and HIV-1 disease progression. Therefore, a better comprehension of the interaction between the receptors of NK cells and their ligands on virally infected cells is required to better understand the role of NK cells in HCV and HIV-1 infections.

c. Receptors

The effector function of NK cells is tightly regulated by a plethora of receptors which allows them to recognize and kill virally infected cells[80]. These receptors are germline-encoded and recognize host molecules, stress-induced ligands and infectious non self-ligands. In addition to the FcγRIIIa receptor (CD16), which can bind to the Fc-region of IgG antibodies, three major families of NK cell receptors control NK cell function: Killer Immunoglobulin Receptors (KIR), Natural Cytotoxicity Receptors (NCR) and CD94/NKG2 C-type lectin receptors[6,121,122]. Each of these receptors bind to specific ligands which can be either presented by the Major Histocompatibility Complex (MHC) class I or directly expressed at the cell surface of target cells. NK cell receptors are generally either activating or inhibitory, defined by their Immunoreceptor Tyrosine-based Inhibitory Motifs (ITIM) (inhibitory) or Immunoreceptor Tyrosine-based Activation Motifs (ITAM) (activating) domains on the intracellular tail of the receptors. NK cell function is tightly regulated by the balance of all the NK cell receptors signals *(Fig I.4.)*

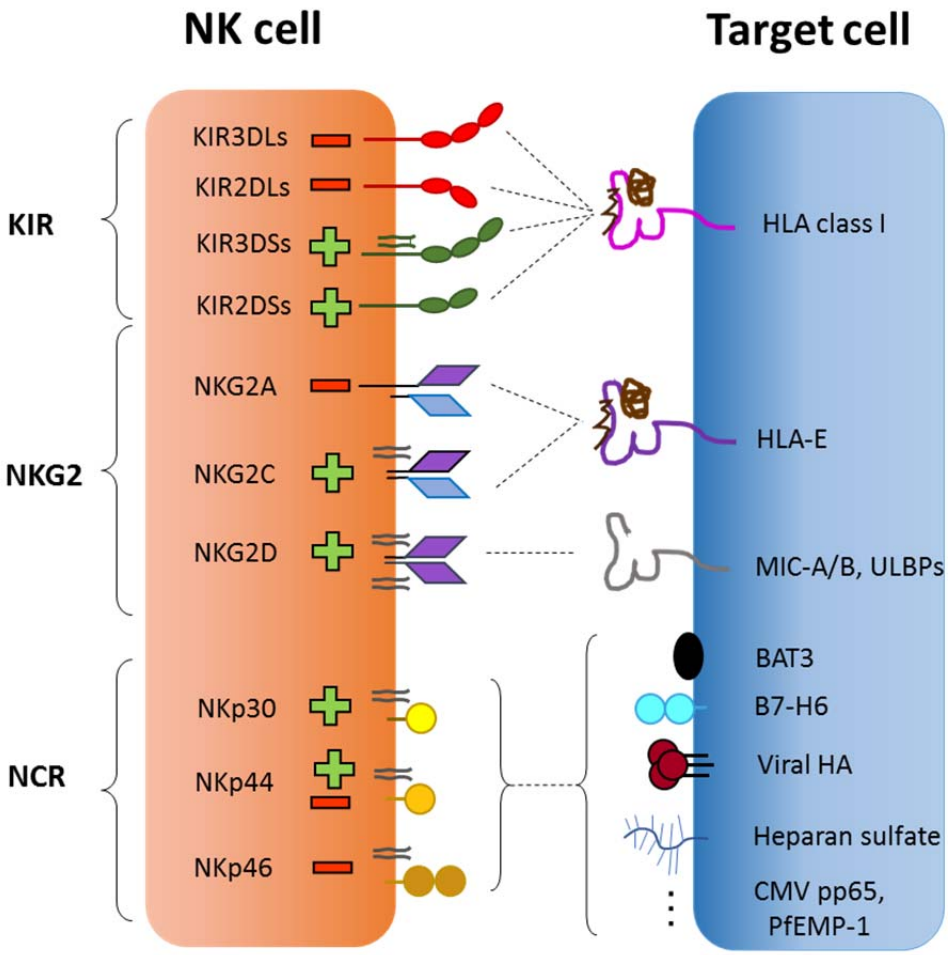

Fig I.4. Major NK cell receptors and ligands (adapted from[123]).

NK cell receptors are constituted by three major families: KIRs, NKG2 and NCRs. Each family contains activating receptors (green plus sign) and inhibiting receptors (red minus sign). Each receptor binds to specific ligands expressed at the surface of the target cell.

d. Mechanisms of NK cell mediated recognition of virally infected cells.

NK cell function is strongly regulated by the integration of signals from inhibiting and activating receptors[6] *(Fig I.5)*. Under homeostatic circumstances, the majority of healthy host cells express MHC class I molecules which bind to inhibitory receptors of NK cells, keeping them inhibited. Studies have shown that the strength of inhibiting signals overcomes the activating signals in a process called "tolerance" *(Fig I.5a)*[124]. During viral infections or malignancies, a panel of common modifications on the target cells are triggered, which are sensed by NK cells. In this case, NK cell activation depends on the loss of inhibitory signals provided by MHC class I molecules, named the "missing-self" hypothesis[121] *(Fig I.5b)* and the expression of stress- or virus-induced ligands for activating NK cell receptors overruling the inhibitory NK cell signals, referred to

"stressed-self" *(Fig I.5c)*[125–127]. Activation of NK cells leads to the elimination of the abnormal cells through cytotoxicity or indirectly through the production of pro-inflammatory cytokines[128].

The interplay between human MHC class I molecules, also named HLA class I, and NK cell receptors binding to HLA class I, is essential to understand how viral replication can be limited by NK cells or at the contrary, how viruses have the ability to escape NK cells´ effector functions. Notably, HCV core protein enhances cell surface expression of HLA class I molecules on HCV-infected cells through triggering p53 expression which upregulates TAP1 production[129]. As ligands for inhibitory NK cell receptors, the upregulation of HLA class I molecules by HCV impairs NK cell function. On the other hand, HIV-1 has developed several escape strategies to avoid NK cell recognition. The HIV-1 accessory protein Nef downregulates the expression of HLA class I molecules HLA-A and HLA-B in an effort to escape $CD8^+$ T cell lysis[130], which however leads to an enhanced NK cell-mediated recognition ("missing-self"). Nef spares (or does not affect) the expression of HLA-C and HLA-E, two major contributors to NK cell inhibition[131,132]. However, a recent study suggests that HLA-C can be downregulated by the HIV-1 Vpu protein of most primary HIV-1 clones although not by the laboratory-adapted NL4-3 virus[133,134], through specific amino-acids in the N-terminal protein Vpu sequence. Additionally, the interaction of HLA class I with NK cell receptors is partially dependent on the HLA class I-presented peptide[135–139]. Overall, this suggests a more complex role of HLA-C, and HLA class I molecules in general, in virus evasion and NK cells recognition. The HLA class I locus is central to NK cell surveillance of potentially stressed or virus-infected cells and a substantial amount of viral evasion mechanisms are targeting HLA class I. The alteration of HLA class I expression on viral infected cells are mainly monitored by the KIRs, one of the main family of NK cell receptors, and will be investigated in more details in this study.

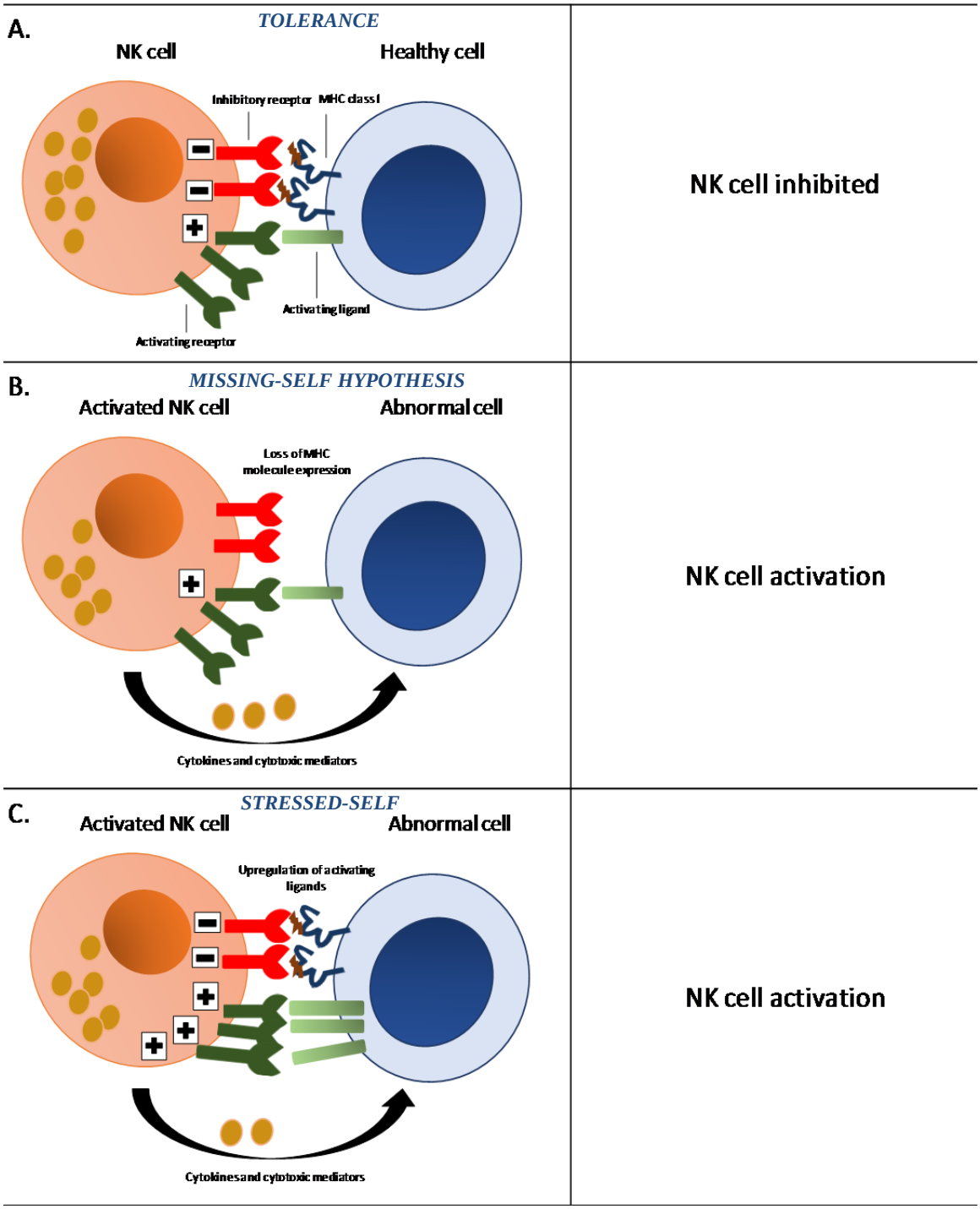

Fig I.5. Recognition of virally infected cells by NK cells (adapted from[140]).

The function of NK cells (activation or inhibition) is determined by the integration of activating and inhibiting signals. Under homeostatic circumstances, the interaction between inhibiting receptors of NK cells and MHC class I ligands of the target cell maintains NK cells inhibited (5.a). NK cells can be activated under two conditions: lack of ligands for inhibiting NK cell receptors (5. b) and/or overexpression of ligands for activating NK cell receptors (5. c).

IV. Impact of Killer-Immunoglobulin like Receptors

Killer-Immunoglobulin like Receptors (KIRs), one of the major families of NK cell family receptors, are type I transmembrane glycoproteins expressed principally on NK cells and some subsets of T cells[141]. KIRs are composed of 14 receptors, either activating or inhibitory, and interact with HLA class I allelic variants expressed on all nucleated cell types[142]. Initially, it was described that NK cells expressed a various number of KIRs on the cell surface in a stochastic manner [143]. Nonetheless, recent data has shown that expression is not entirely random but rather that an educational process shapes the KIR repertoire to influence NK-cell functionality and to maximize the balance between effective defense and self-tolerance[144]. In addition, genes encoding for the KIR family are highly polymorphic and various alleles of the same KIR are able to bind to MHC class I molecules with different affinity[145,146]. In humans, two different types of KIR haplotypes evenly distributed within the population are described, A and B, which mainly differ in the content of activating KIRs. Indeed, while both haplotypes share inhibitory KIRs, haplotype A includes a single activating KIR (KIR2DS4) whereas haplotype B includes up to five activating KIRs[147] *(Fig I.6)*. Altogether, a varied pattern of KIR-expression exists among individuals, allowing for a diverse NK cell repertoire able to sense changes in MHC class I expression.

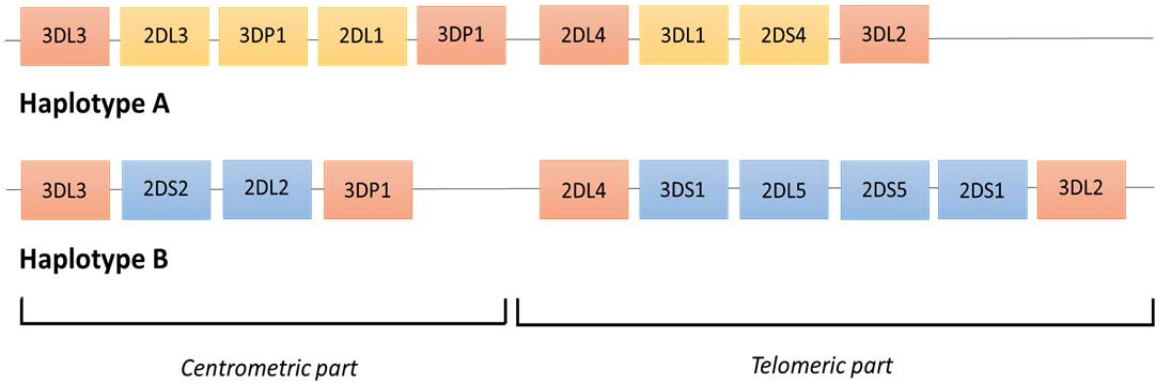

Fig I.6. Representative organization of the human KIR locus (adapted from[142,148]).

Two KIR haplotypes have been described depending on the gene content. The two KIR haplotypes, named haplotype A and haplotype B, share inhibitory KIRs but differ in the content of activating KIRs. Haplotype A contains only a single stimulatory KIR gene, KIR2DS4, whereas haplotype B contains various combinations of KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5, KIR3DS1, and KIR2DS4. Conserved genes are shown in red and are virtually present on all haplotypes. Genes that can be present in both haplotype A and haplotype B are shown in yellow, and genes that are specific to haplotype B are shown in blue.

a. Molecular Structure

While KIRs are characterized to be extensively genetically variable, all KIRs share a similar molecular structure consisting of a type 1 transmembrane glycoprotein with two or three extracellular domains (KIR2D or KIR3D) and one long (L) or short (S) cytoplasmic tail. Inhibitory KIRs have a long cytoplasmic tail bearing ITIMs, defining them as KIR2DL or KIR3DL[142]. Conversely, activating KIRs have a short cytoplasmic tail which transmits activating signals through the interaction of Immunoreceptor Tyrosine-based Activation Motifs (ITAMs) with adaptor molecules such as DAP12[142] , defining them as KIR2DS or KIR3DS *(Fig I.7.)*.

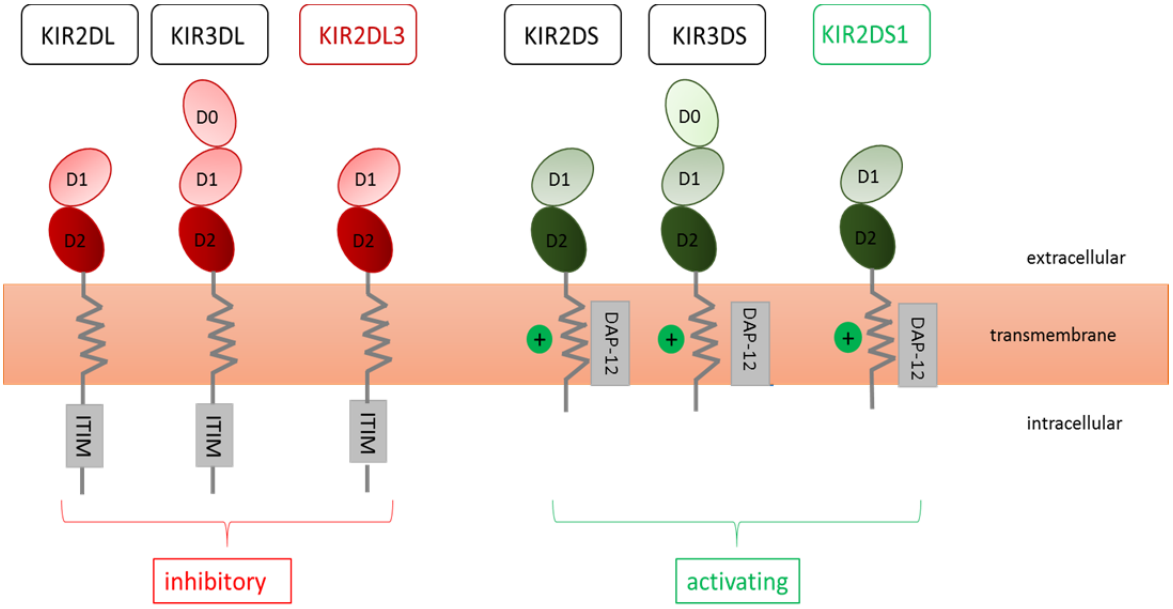

Fig I.7. Structure of KIR receptors.

KIR receptors contain two (KIR2D) or three (KIR3D) extracellular domains and can be inhibiting (red) or activating (green) depending of the length of their cytoplasmic tail and the ITIMs or ITAMs motifs associated. KIR2DL3 and KIR2DS1 are two representative examples of KIRs and will be studied in more details in the rest of the thesis.

b. KIR-Ligands

The majority of KIRs interact with specific allotypes of HLA class I molecules[142]. HLA class I molecules are glycoproteins expressed on the surface of nucleated cells. The HLA class I structure consists of two polypeptides chains: α and β2 microglobulin (β2m) noncovalently associated. The α chain is encoded by the HLA gene and can be divided in three domains: α1, α2 and α3. The α1 and α2 domains folded together into a single

structure to form a groove, which is the site where peptides bind to HLA molecules. The α3 domain is an immunoglobulin-like domain spanning the membrane[149,150]. HLA class I molecules are divided in two categories: classical HLA class I (HLA A, B and C) and non-classical HLA class I (HLA E, F and G)[151]. In general, receptors of the KIR3D group engage HLA-A and HLA-B while KIR2D receptors interact with HLA-C molecules[152]. HLA-C can be further subdivided into two groups: HLA-C group 1 (HLA-C1), characterized by an asparagine in position 80, binding to KIR2DL2 and KIR2DL3 [153], and HLA-C group 2 (HLA-C2), characterized by a lysine in position 80, preferentially binding to KIR2DL1[154] *(Table I.1)*. Some described KIRs still lack identified ligands; in particular, the specificities of most activating KIRs remain elusive. Indeed, KIR2DS1 has been described to bind HLA-C2, but with low affinity[155]. KIR2DS2 has been shown to bind to HLA-C1 molecules and also with low affinity[156] and to HLA-A*11[157]. KIR3DS1 has been very recently described to bind to HLA-F open conformers[158], whereas the ligands for KIR2DS3 and KIR2DS5 remain unknown.

Table I.1. KIR receptors and their ligands[45,159,160].

KIR	Ligand
KIR2DL1	HLA-C2
KIR2DL2/L3	HLA-C1
KIR2DL4	HLA-G
KIR2DL5	unknown
KIR3DL1	HLA-Bw4
KIR3DL2	HLA-A*03/A*11, HLA-F
KIR3DL3	unknown
KIR2DS1	HLA-C2 (weak)
KIR2DS2	HLA-C1 (weak), HLA-A*11:01

KIR2DS3	unknown
KIR2DS4	HLA-A*11:02, HLA-C*05:01, HLA-C*16:01
KIR2DS5	unknown
KIR3DS1	HLA-F open conformers

c. HLA class I-presented peptides

The structural principles that allow KIRs to interact with their cognate HLA class I molecules have been addressed by several studies using crystal structures of KIR2DL1[154], KIR2DL2[161], KIR2DL3[162] and KIR2DS2[157]. These studies showed that KIRs not only interact with motifs located in the heavy chain of HLA class I molecules but also directly recognize the C-terminal end of the HLA-presented peptide. Therefore, the HLA-presented peptide has the ability to modulate the KIR/HLA interaction.

Peptides presented by HLA class I molecules are generally short sequences of Amino Acids (8-11 AA) originating from self, altered or viral molecules[163]. Presented peptides are mostly generated by proteasomes in the cellular cytosol and loaded to HLA class I molecules in the ER with the help of the Peptide Loading Complex (PLC). The PLC consists of the HLA class I, the oxidoreductase ERp57, the chaperone molecules Calereticulin and Tapasin and the Transporter associated with Antigen Processing (TAP) which translocates the peptides from the cytosol to the ER lumen[163]. After assembly and loading of the HLA class I molecule, the complex move to the cell surface through the secretory pathways passing the Golgi apparatus[163]. Of note, several Post-Translational Modifications (PTMs) occur during the antigen processing pathway including phosphorylation, glycosylation, alternative splicing, oxidization and lipidation[164,165].

Under homeostatic conditions, 10^3-10^4 HLA class I-presented peptides have been calculated to be expressed per cell[166]. The presence of peptides is essential for correct folding, expression and function of HLA class I molecules[167]. HLA-presented peptides are not only described for their KIR interaction, but primarily defined for their interaction with T Cell Receptors (TCR). However, while TCRs interact with peptides at the central

peptide positions P4-P6[168], KIRs interact principally with the C-terminus of the peptide near positions P7-P8[169]. The impact of HCV and HIV-1 infection on HLA class I presented epitopes has been particularly studied in the context of recognition by $CD8^+$ T cells[170,171]. Nevertheless, several studies have shown that changes in HLA class I-presented peptides occurring during HIV-1 impact also KIR interaction by either compromising or promoting KIR binding affinity and thus modulates NK cells functions[136,139,172–174]. On the contrary, the impact of HLA class I HCV-presented-peptides on KIR interaction has not been yet studied and need further investigations.

V. Influence of KIR/HLA associations on disease outcome

The clinical outcome of HCV and HIV-1 infections varies substantially between individuals, mostly due to host genetics factors[8,9]. The identification of several KIR/HLA combinations associated to differential outcomes in HCV and HIV-1 disease progression suggested the KIR/HLA class I interactions might account partly for the observed variation in inter-individual disease outcome.

In particular, several studies identified *KIR2DL3* association with its ligands *HLA-C1* as a protective combination in the context of HCV clearance in intravenous drug users[84,85]. The specific *KIR2DL3/HLA-C*03:04* association also predominates in HCV-exposed seronegative aviremic individuals[86] and in HCV-infected individuals with sustained responses to IFN-α-based therapies[175]. Additionally, *KIR2DS3/HLA-C2* was identified as a risk factor for the development of chronic HCV infection[176,177]. The expression of *KIR3DS1* in combination *HLA-Bw4-80I* has also been suggested to be protective in the context of hepatocellular carcinoma development in patients with chronic HCV infection[178]. The combined expression of *KIR3DL1* with *HLA-Bw4-80T* was furthermore described recently to be associated with spontaneous clearance of HCV infection in a cohort of people who inject drugs in North America[179].

In HIV-1 infection, the combination *KIR3DS1/KIR3DL1* and *HLA-Bw4-80I* was shown to significantly impact HIV-1 disease outcome[9]. Indeed, HIV-1-infected individuals expressing *HLA-Bw4-80I* in combination with *KIR3DS1*[108] or *KIR3DL1*[180] showed a slower progression to AIDS without HAART. $KIR3DS1^+$ NK cells have also a stronger activity in HIV-1-infected individuals encoding for -Bw4 alleles[181] and strongly inhibit HIV-1 *in vitro*[109]. Additionally, two independent Genome-Wide Association Studies

(GWAS) have identified an association between a better control of HIV-1 and the Single Nucleotide Polymorphism (SNP) rs9264942 located 35kb upstream of HLA-C[182,183]. The SNP rs9264942 is associated with higher level of HLA-C transcription and cell surface expression[184,185]. Furthermore, HLA-C serves as ligands for KIR2D receptors and variations in HLA-C surface expression might influence NK cell functions. Altogether, an increasing number of disease association studies implicate distinct KIR/HLA interactions in shaping the course of HIV-1 and HCV infections. However, the underlying mechanisms explaining how the KIR/HLA modulates the outcome of HIV-1 and HCV are not fully understood.

a. KIR2DL3/HLA-C*03:04

First described in 1995, KIR2DL3 is an inhibitory receptor with two extracellular domains[162] *(Fig I.7)* binding to HLA-C1 molecules (HLA-C*01/*03/*07/*08/*12/*14/*15:07/*16:01)[142]. KIR2DL3 is expressed by 75-100% of humans[186] and is present on both haplotypes A and B *(Fig I.6)*. Although KIR2DL3 alleles are highly polymorphic[187], most of the studies focus on *KIR2DL3*001*, the most common allele in European and North-American populations[188]. KIR2DL3 and KIR2DL2 are alleles of a single locus[189]and share the same HLA-C1 ligands although with different binding affinity, with KIR2DL2 described as a stronger receptor for HLA-C1 than KIR2DL3[190].

Several genetics studies have found association of KIR2DL3 with various disorders, particularly with viral infections[84–86,175,191–195] and autoimmunity[196–199] *(Table I.2)*.

Table I.2. Most common disorders associated with KIR2DL3.

Disorders	Description of KIR2DL3 disorders association
Viral infection	
HCV	Better clearance of HCV in individuals when associated with HLA-C1[84–86]. Better response to treatment (pegylated alpha interferon and ribavirin) when associated with HLA-C1.[175,200] Non-response to treatment in chronic HCV genotype 3a when associated with HLA-C1/C1[192]
HIV-1	Better immunological response to cART when associated with HLA-C1[194] Resistance to HIV-1 in female sex-workers in the absence of HLA-C1[193]
Tuberculosis Pulmonary	Increased susceptibility[195,201]
Autoimmunity	
Diabetes	Increased susceptibility when associated with HLA-C1[196,197,202,203]

Melitus 1	
Vogt-Koyanagi-Harada	Protective when associated with HLA-C1[198,199]

The KIR2DL3 crystal structure revealed that KIR2DL3 binding to its HLA-C1 ligand is peptide-dependent[162]. *In vitro* studies confirmed KIR2DL3 peptide selectivity by identifying peptides variants presented by HLA-C*01:02 which tightly modulates KIR2DL3 binding[136,204] and thus impact KIR2DL3$^+$ NK cell function[205]. Furthermore, HIV-1 p24 (GAG) peptides presented by HLA-C*03:04 have been recently identified for their ability to impact KIR2DL3 binding and NK cell function[139]. To our knowledge, no HCV peptides have been identified yet despite the strong association between HCV control and KIR2DL3.

b. KIR2DS1/HLA-C*06:02

KIR2DS1 is an activating receptor with two extracellular domains *(Fig I.6.)* belonging to the KIR haplotype B[142] *(Fig I.7)*. KIR2DS1 is expressed worldwide in the human population at various frequencies depending on the geographic distribution, ranging from 14% to 88%, and specifically at a 50% rate within the European population[186]. KIR2DS1 is relatively conserved[187] and *KIR2DS1*002* is described as the most frequent allele in the human population[206]. A growing number of genetic studies have identified associations between the presence of the activating KIR2DS1 receptor and susceptibility to autoimmune diseases[207–209], reproductive success[210,211], control of viral infections[195,212] and malignancy in cancer[213–215] *(Table I.3)*. Of note, KIR2DS1 is often described to play an important role in pregnancy[216]. During pregnancy, uterine NK cells (uNK cells) represent the majority of leukocytes in the decidua, the uterine endometrium in pregnancy corresponding to the maternal part of the placenta[217]. By interacting with fetal ExtraVillous Trophoblasts (EVT), uNK play an essential role in remodeling maternal arteries (also called spiral arteries), allowing for the correct supply of oxygen and nutrients to the placenta[218,219]. An incorrect supply to the placenta will lead to babies with smaller weight, reducing neonatal survival rates and increasing the risks of pre-eclampsia and IntraUterine Growth Restriction (IUGR)[216]. KIR2DS1 expression on uNK cells has been linked to implantation and pregnancy success[210]. Indeed, when the fetus expresses HLA-C2$^+$ ligands binding to KIR2DS1$^+$ uNK cells, it leads to an increased production of cytokines by the KIR2DS1$^+$ uNK cells, enhancing EVTs invasion, improving oxygen and

nutrient supplies[210]. On the contrary, a mother carrying a haplotype A/A and, therefore, not expressing the KIR2DS1 receptor, has an increased risk to develop pre-eclampsia and IUGR if the fetus is HLA-C2^{+220}. Altogether, these studies strongly suggest that KIR2DS1 receptor expression on uNK cells might be linked to reproductive success, indicating a potential tissue-specific role for this activating KIR. However, the precise ligands and mechanisms that regulate KIR2DS1^{+} NK cells and their role in viral infections are not well characterized.

Table I.3. Most common disorders associated with KIR2DS1.

Disease	Description of KIR2DS1 disorders association
Viral infections	
Ebola	Associated with fatal outcome[212].
Tuberculosis (TB)	Increased susceptibility[195].
Autoimmunity	
Psoriasis vulgaris (PV)	Increased susceptibility when associated with HLA-C*06:02[207,208,221,222]
Psoriasis arthritis (PA)	Increased susceptibility in the absence of HLA-C2 alleles[209,223,224].
Scleroderma	Increased susceptibility[225,226].
Systemic Lupus Erythematosus (SLE)	Increased susceptibility[225,227]
Ankylosing Spondylitis (AS)	Increased susceptibility in association with HLA-C2[228,229].
Reproduction associated troubles	
Preeclampsia, Fetal Growth Restriction (FGR)	Reduced risk when the fetus is HLA-C2[210,220,230,231]
Recurrent Spontaneous Abortion (RSA)	Reduced risk when the fetus is HLA-C2[231], Increase risk when the women is homozygous for HLA-C1 and the fetus is HLA-C2^{+211} .
Cancer	
Chronic Myoloid Leukemia (CML)	Increased susceptibility[213], absence of KIR2DS1 associated with better response to Imatinib treatment[214].
Melanoma	Increased susceptibility in the absence of HLA-C2 ligands[215].
Other	
Allogeneic stem cell transplantation	Reduced risk of Leukemia relapse for HLA-C2 patient if the donor is HLA-C1[232]

KIR2DS1 ligands are generally described as HLA-C2 molecules (HLA-C*02/*04/*05/*06/*07:07/*12:042/*15/*16:02/*17)[155,233–235]. The described binding is often weak[155], especially in comparison to KIR2DL1, the inhibitory counterpart of KIR2DS1. KIR2DS1 and KIR2DL1 share a high degree of sequence homology[236] and bind to the same HLA-C2 ligands[233]. To explain the different binding affinities to their ligands, HLA-C2 presented peptide recognized by KIR2DS1 may potentially play an important role. The crystal structure of KIR2DL1 in association with the HLA-C2 allele

HLA-C*04:01 has demonstrated that KIR2DL1 binding is peptide dependent[154] . However, no crystal structure is available for KIR2DS1. One HLA-C*04:01 presented peptide has been described to modulate KIR2DS1 binding[155,235] although the functional consequences of this interaction remain unclear and no viral peptide has been described so far. Altogether, the functional relevance of the KIR2DS1 binding to HLA-C2 is puzzling and needs to be further investigated.

VI. Aims

NK cells play an important role in viral infection and distinct KIR/HLA associations have shown to influence viral disease outcomes, in particular for HCV and HIV-1 infection. Viral infections such as HCV and HIV-1 infection are able to alter the repertoire of peptides presented by HLA class I molecules and thereby impact KIR/HLA interactions. Based on the fact that viral peptides presented by HLA class I molecules modulate KIR binding and influence NK cell function, this thesis sought to address the following aims:

1) Identification of HCV peptides presented by **HLA-C*03:04, which influence KIR2DL3 binding and KIR2DL3$^+$ NK cell function.**

2) Identification of **peptides modulating the KIR2DS1/HLA-C*06:02 interaction** and their impact on **the function of KIR2DS1+ NK cells.**

PAPERS

I. Sequence variations in HCV core-derived epitopes alter binding of KIR2DL3 to HLA-C∗03:04 and modulate NK cell function. ***(Chapter 1, Appendix)***

II. Peptide-specific engagement of the activating NK cell receptor KIR2DS1. ***(Chapter 2, Appendix)***

DISCUSSION

Persistent human viral infections, such as HCV and HIV-1, are characterized by a constant struggle between antiviral immunity and viral escape from immune control. Early immune responses against HCV and HIV-1 have shown to be crucial to contain virus replication or prevent the development of chronic viral infection[45]. NK cells, the main effector innate immune cell subset, play an important role in the early stages of viral infections. The repertoire of peptides, or peptidome, presented by HLA class I molecules is altered during viral infections[237]. Although HLA class I-presented peptides are mainly studied for their interaction with TCRs[238], an impact on the KIR of NK cells has also been demonstrated[169]. Indeed, specific KIR/HLA associations have shown to influence HCV and HIV-1 disease outcome[8,9]. Therefore, this work hypothesizes that KIR^+ NK cells are able to monitor alterations in the peptide repertoire presented by HLA class I. The aim of this thesis was to identify peptides binding to specific KIR receptors and modulating KIR^+ NK cells function.

I. Characterization of the HLA class I-presented peptides binding to KIR2DL3 and KIR2DS1.

The interaction between NK cells and infected cells is characterized by a complex interplay between inhibitory and activating receptors on the surface of NK cells and several HLA- and non-HLA ligands on the surface of the infected cells. Therefore, to identify HLA class I- presented peptides involved, respectively, in KIR2DL3 and KIR2DS1 binding, we developed a simplified cellular model consisting of TAP-deficient cell lines transfected with individual HLA class I alleles and either KIR-IgG Fusion construct or reporter cell lines transfected with individuals KIRs *(Figure 1, Appendix)*. This model allowed us to study the interaction between one single peptide-HLA class I complex and one single KIR molecule.

a. Characterization of peptides stabilizing HLA-C*03:04 and HLA-C*06:02.

Large screening of peptide libraries allowed us to identify specific amino acid sequences binding to HLA-C*03:04 and HLA-C*06:02, respectively.

For HLA-C*03:04, 12 strongly binding peptides were identified to stabilize HLA-C*03:04, consisting of peptides between 9 and 15 Amino Acid (AA) length from HCV genotype 1 Core and NS3 (*Chapter 1, and Table 1 Appendix*). The described optimal binding motif for HLA-C*03:04 peptide presentation is between 9-11 AA and consists of a strong anchor at position 2 (P2) containing Alanine (A) or Serine (S) and a Leucine (L) or Methionine (M) in P9[239,240]. Our results showed discrepancies with the previously published optimal size[240] and binding motifs[239] *(Fig III.1)*. However, the number of peptides tested in our library was limited as it consists of 200 overlapping peptides of 15 amino acid length *(Chapter 1, and Table 1 Appendix)* and 10 peptides of 9 amino acid length optimized from the previous identified peptides *(Chapter 1)*. Therefore, no further conclusion could be drawn concerning optimal binding motifs for HCV peptides stabilizing HLA-C*03:04. Further investigations will be required to perform a complete mapping of HLA-C*03:04 epitope.

Concerning HLA-C*06:02, a library of 568 overlapping peptides spanning the entire HIV-1 clade B sequence (346 peptides with length of 18 AA covering the entire HIV-1 consensus sequence and 222 decametric peptides overlapping by 9 amino acid and covering p24 GAG) were assessed for their potential to stabilize HLA-C*06:02 (*Chapter 2 and Table 3, Appendix*). 19 synthetic peptide nonamers previously described to bind to HLA-C*06:02 were also tested[241] (*Chapter 2 and Table 2 Appendix)*. In total, 20 peptides were identified to stabilize HLA-C*06:02 surface expression, consisting of 14 synthetic peptides and 6 HIV-1 peptides deriving from p24 Gag, gp120 and RT of HIV-1 clade B. The length of the majority of peptides binding to HLA-C*06:02 was 9 AA (19/20), consistent with literature[242]. Peptides from our library binding to HLA-C*06:02 shared a specific binding motif consisting of a Phenylalanine (F) in P1, an Arginine (R) in P2 and an aliphatic amino acid such as Valine (V), Leucine (L) or Isoleucine (I) in P9 of the peptide sequence *(Fig III. 1b)*. It suggests that P1, P2 and P9 are the anchor residues of the peptide to HLA-C*06:02 and are necessary for the correct binding, which is in agreement with previous literature[239,241,243].

Altogether, our results suggest that HLA-C*06:02 has a specific binding sequence motif among different peptide origins, whereas further investigations are needed to identify a specific peptide binding motif for HLA-C*03:04 presentation of HCV peptides.

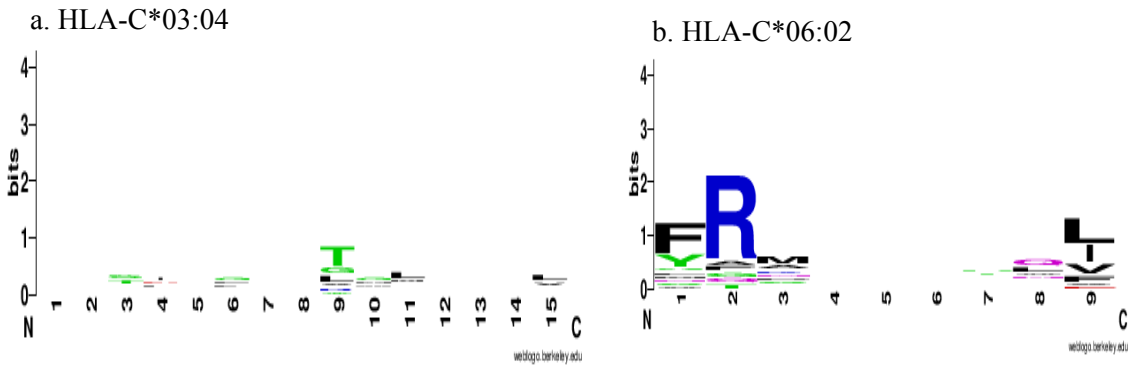

Fig III. 1 Potential binding motif for HLA-C*03:04 and HLA-C*06:02 extrapolated from peptides identified in Chapters 1 and 2.

*Binding motifs are represented as logos generated from the peptides identified as strong binders for HLA-C*03:04 (n=12) and HLA-C*06:02 (n=20) using the website http://weblogo.berkeley.edu/logo.cgi. The size of the letter correlates with the frequency of the AA at the indicated position.*

b. Binding of HLA-C/peptide complexes to KIR2DL3 and KIR2DS1

Strong HLA-binding peptides were further studied for their capacity to interact with KIR2DL3 and KIR2DS1. KIR2DL3 was of particular interest for the study of HCV peptides presented by HLA-C*03:04 as genetics studies showed a correlation between HCV clearance and the combined presence of the alleles *KIR2DL3* and *HLA-C*03:04*[86]. Moreover, this thesis aimed to investigate the peptide specificity of the activating NK cell receptor KIR2DS1 for which precise ligands and functions in viral infections remained unclear. For each KIR, one HLA class I/peptide complex was identified. The viral peptide "YIPLVGAPL" derived from the HCV genotype 1 $Core_{136\text{-}144}$ presented by HLA-C*03:04 showed some remarkable binding to KIR2DL3 and was further demonstrated to inhibit $KIR2DL3^+$ NK cell degranulation *in vitro (Chapter 1)*. In addition, the synthetic peptide "SRGPVHHLL" presented by HLA-C*06:02 activated $KIR2DS1\zeta^+$ Jurkat reporter cell lines as well as $KIR2DS1^+$ primary NK cell clones, resulting in an increased degranulation in comparison to $KIR2DS1^-$ primary NK cell clones *(Chapter 2)*. In summary, these results demonstrate that specific peptides presented by HLA-C*03:04 and HLA-C*06:02 can efficiently mediate binding to KIR2DL3 and KIR2DS1 respectively, consistent with previous findings[139,155,174,205]. Our results showed for the first time that HLA-C*06:02-presented peptides influence the functionality of $KIR2DS1^+$ NK cells and confirm previous studies showing the same effect on HLA-C*03:04-presented peptides and $KIR2DL3^+$ NK cells[139,205].

In contrast to T-cell receptors, which are highly specific to HLA class I-presented peptides[244], KIRs are mostly described to recognize a broader range of peptide-HLA class

I combinations. They are more sensitive to specific groups of amino acids based on their physico-chemical properties (eg: non polar, polar, basic, acidic) rather than to the specific peptide sequence[245]. Indeed, crystal structures of KIR2DL1[154], KIR2DL2[161], KIR2DL3[162] and KIR2DS2[157] in complex with their HLA classI ligand have described that KIRs bind to the α1 and α2 helix of HLA class I and make direct contact with the C-terminal end of the bound peptide, corresponding to P7-P9 peptide residues. Therefore, the amino acid substitutions at P7 for the HLA-C*06:02-presented peptide "SRGPVHHLL" was studied and confirmed that mutations in P7 did not affect HLA-C*06:02 stabilization but were sufficient to modulate KIR2DS1 binding by either increasing or decreasing KIR2DS1ζ^+ Jurkat reporter cell activity *(Chapter 2)*. Moreover, amino acid substitutions on P2 and P9, positions, defined as anchor residues for HLA-C*06:02, also negatively impacted KIR2DS1 binding (*Chapter 2)*. Modifications of these residues might induce conformational changes of the "SRGPVHHLL" peptide presented by HLA-C*06:02, which can abrogate the binding to KIR2DS1. Future studies focusing on decrypting the crystal structure of KIR2DS1 in conjunction with HLA-C molecules presenting specific peptides will help to specifically clarify these interactions.

Altogether, we confirmed the functional impact of HLA class I presented peptide on KIR$^+$ NK cells and identified one HCV-derived peptide presented on HLA-C*03:04 "YIPLVGAPL" which modulates KIR2DL3$^+$ NK cell function and one synthetic HLA-C*06:02 presented peptide "SRGPVHHLL" modulating KIR2DS1$^+$ NK cell function. Despite a large screening of libraries of viruses and synthetic peptides, we could no longer detect virus-derived peptide for engaging binding of KIR2DS1. Indeed, the peptide "SRGPVHHLL" identified to enable strong KIR2DS1-binding to HLA-C*06:02 was derived from a synthetic peptide library predicted to bind to HLA-C*06:02[241], and does not match any known viral or human epitope. Further implications will be discussed in chapter II.

c. Limitations of the cellular model

Our study used a reductionist system to explore how single HLA class I peptides impact KIR$^+$ NK-cell recognition and function. One limitation is the fact that the peptides studied are not naturally processed. We used cell lines with knock-out for TAP1, preventing loading of self-peptide to HLA class I molecules and allowing HLA molecules to be pulsed externally with peptides[246]. However, the Post Translational Modifications (PTM)

occurring during the antigen processing pathway such as phosphorylation, glycosylation, alternative splicing, oxidization and lipidation[164,165] might modulate KIR recognition and NK cell function. Indeed, differential glycosylation was already shown to impact KIR recognition as alteration of the N-glycan from the HLA-B*57:01 molecule was demonstrated to affect KIR3DL1 recognition[247].

Another limitation of our study is that the HLA class I proteome monitored by T cells and NK cells does not only consist of viral peptides but also of stressed, altered self-peptides (immunopeptidome)[248,249]. Viral infections impact the presentation of self-peptides on several ways including switching off host protein synthesis, interfering with HLA class I peptide loading, and/or HLA class I cell surface expression by altering its recycling pathway[237,246,250,251], augmented rate of Defective Ribosomal Products (DRiPs)[252], cellular redox state increase and oxidative presented-peptide modifications [253]. Furthermore, the analysis of HLA class I proteome by Mass Spectrometry (MS) on HLA-B*0702 eluted peptides from HIV-1 infected cells revealed the presence of host-derived epitopes uniquely presented on HIV-1 infected cells[254] strongly suggesting that self-peptides epitopes impact TCR and possibly KIR recognition. The present study therefore is limited in that it cannot address the kinetics of viral peptide HLA class I presentation as well as the overall alteration of HLA class I presented self-peptides induced by viral stress. Consequently, ongoing studies in our laboratory are focusing on studying the altered self-HLA class I proteome binding to HLA-C*03:04 and HLA-C*06:02 following HCV and HIV-1 infection respectively via MS, which will help to identify novel and naturally processed HLA classI/peptide complexes involved in KIR2DL3$^+$ and KIR2DS1$^+$ NK cell recognition.

Recent studies suggest that a mixture of peptides with different binding affinities can impact NK cell recognition, suggesting that NK cells would sense changes in HLA class I peptide repertoires rather than specific HLA class I/peptide complexes[136,255]. This hypothesis, termed peptide antagonism, has been studied on the binding of HLA-C*01:02-presented peptides to two inhibitory KIRs (iKIRs), KIR2DL3 and KIR2DL2. It suggests that HLA class I-presented peptides binding to specific KIRs can be divided in two categories based on their affinity for iKIR$^+$ NK cells: weak and strong peptide binders. Weak HLA class I-presented peptides binding to iKIRs might act as altered peptide ligands for iKIRs and thus weaken the NK cell-target cell interaction, leading to NK cell activation. Rather than a simplified model where either individual HLA class I-

presented peptides bind or not to iKIR$^+$ NK cells, the peptide antagonist hypothesis suggests a complex model where peptides with different binding affinities act synergistically to impact iKIR$^+$ NK cell function. However, this hypothesis was applied to iKIRs and has not been studied in the context of activating KIRs (aKIRs) to date.

II. KIR$^+$ NK cell-mediated immune pressure on HCV and HIV-1

a. HCV might escape from KIR2DL3$^+$ NK cell-mediated immune pressure.

The HLA-C*03:04-presented peptide "YIPLVGAPL" identified in this study for its ability to induce KIR2DL3$^+$ NK cell inhibition is derived from the core protein sequence of HCV genotype 1. The HCV genotype 1 accounts for approximatively 40-50% of HCV cases worldwide (*Chapter 1*). Interestingly, several naturally occurring sequence variations of this epitope in other HCV genotypes (genotype 6, 3+4 and 2) exhibited reduced capacities to bind to KIR2DL3 and a lower ability to inhibit KIR2DL3$^+$ NK cells function (*Chapter 1*). These results hint at a potential escape mechanism of HCV genotype 1 from NK cell-mediated immune pressure through the presentation of viral epitopes engaging iNK cell receptors *(Fig. III. 2)*.

Within an HCV-infected individual, HCV does not only exist as a single virus genome, but rather as a cloud of viruses with various mutations leading to high genome diversity. This concept is also called virus quasi-species[256] and suggest that single viral sequences are targeted by the immune response driving viral evolution. HCV viral quasi-species, therefore, might lead to a diversity of variable epitopes presented by HLA class I. In the presence of immune selection pressure exerted by iKIR$^+$ NK cells against the Wild-Type (WT) viral sequence, the mutant progeny encoding altered epitopes enhancing binding to iKIR$^+$ NK cells would preferentially expand in infected individuals.

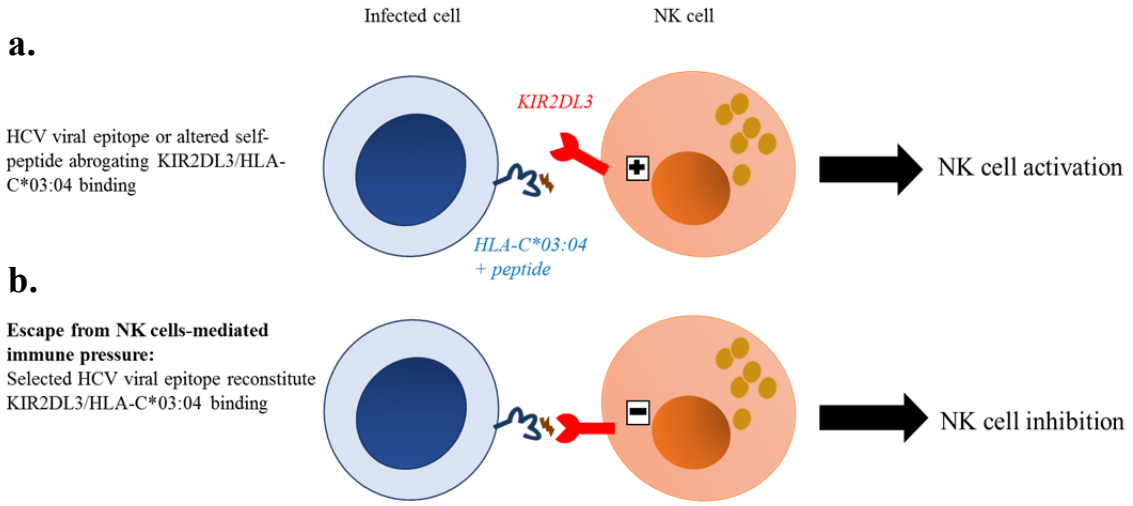

Fig III. 2 Simplified model displaying HCV escape from KIR2DL3$^+$ NK cells-mediated immune pressure (adapted from[45,257]). *(a) In the setting of HCV infection, HLA-C*03:04 presents an increased number of viral epitopes or altered self-peptides which does not allow for KIR2DL3 binding and results in NK cell activation.* (b) *We hypothesize that HCV can select for epitope variants reconstituting KIR2DL3/HLA-C*03:04 binding and leading to NK cell inhibition.*

Our study is the first to suggest an escape mechanism from HCV to iKIR$^+$ NK cells (*Chapter 1*). This escape mechanism has already been described in the context of HIV-1 and SIV infections[139,172–174,258–260]. The ability of HCV to select specific epitope variants escaping CD8$^+$ T cell recognition has been well characterized[261–265]. One of the first studies, performed in 8 chronically infected chimpanzees, described CD8$^+$ T cell escape in a single epitope, linking CD8$^+$ T cell selection pressure to the genetic composition of HCV quasi species[264]. In humans, several studies have been later performed and confirmed an HCV mutational escape from CD8$^+$ T cell immunity[261–263,265]. Tester *et al.* studied two individuals acutely infected with the same HCV strain but developing divergent clinical outcomes, one resolving spontaneously and the other one progressing towards chronical HCV infection. An escape mutation in an immuno-dominant epitope was observed in the individual who did not spontaneously resolved the infection, indicating the importance of immunological pressure [262]. Simultaneously, another group followed a cohort of injection drugs users during the first year after HCV infection and described the presence of amino acid substitutions in CD8$^+$ T cells epitopes in individuals which did not clear viremia[265]. In summary, the ability of HCV to escape CD8$^+$ T cell-mediated immune pressure has shown to be linked to viral persistence and clinical outcome in infected individuals.

KIR2DL3 and HLA-C1 are described as a protective combination in the context of HCV viral clearance[84]. Additionally, the interaction of KIR2DL3 with its HLA-C1 ligand is described to be more peptide selective than other iKIRs such as KIR2DL1[266]. Consequently, KIR2DL3$^+$ NK cells might exert a strong immune pressure on HCV virus. HCV may escape KIR2DL3$^+$ NK cells-mediated immune pressure through the selection of epitopes strengthening the binding of HLA-C1 to KIR2DL3, which would consequently increase the inhibition of KIR2DL3$^+$ NK cell function. One limitation is, however, that our results were performed with KIR2DL3$^+$ NK cells derived from Peripheral Blood Mononuclear Cells (PBMCs). Since recent publications have demonstrated that intrahepatic NK (ihNK) cells display distinct functional features[267–269], follow-up studies will need to focus on confirming our results with tissue-derived NK cell populations.

One of the first studies describing viral escape from NK cell-mediated pressure was performed in the HIV-1 model and identified several amino acid polymorphisms significantly associated with the presence of specific KIR genes[258]. In particular, viral sequences polymorphisms in a region encoding for an overlapping segment spanning the carboxyterminal (C-term) end of *Vpu* and aminoterminal (N-term) end of *Env,* named in the study *Vpu-Env*$^{V/V}$, were described to be significantly enriched in individuals encoding for inhibitory *KIR2DL2*[258]. Functionally, these escape variants enhanced the ability of KIR2DL2$^+$ NK cells to bind HIV-1-infected cells and reduced the antiviral activity of KIR2DL2$^+$ NK cells *in vitro*[258]. subsequently *in vitro* studies have furthermore highlighted that sequence variants within *p24 (gag HIV-1)* derived epitopes presented by HLA-C*01:02 or HLA-C*03:04 altered KIR2DL2 and KIR2DL3 binding and modulated KIR2DL2/3$^+$ NK cell function[172,174]. Additionally, in a cohort of 406 individuals chronically infected with HIV-1 clade C (from Durban, South Africa), a viral sequence variant in HIV-1 clade B, $T_{gag303}V$, was significantly enriched in individuals encoding for the combination *KIR2DL3/HLA-C*03:04*[139]. Functionally, this sequence variant enabled stronger binding to KIR2DL3 when presented by HLA-C*03:04 and led to a reduced degranulation of KIR2DL3$^+$ NK cells against variant peptide-loaded target cells expressing HLA-C*03:04 compared to the HLA-C*03:04 presented wild type epitope. These results suggest that HIV-1 may escape NK cell-mediated immune pressure through the selection of KIR/HLA-associated sequence polymorphisms impairing KIR$^+$ NK cell function.

While NK cells are described to expand first in acute viral infection[45] and could drive escape mutations, NK cells and HIV-1 specific $CD8^+$ T cells were suggested to expand and respond both with similar kinetics during chronic HIV-1 infection[270]. In addition, KIRs are also expressed on T cells and can modulate $CD8^+$ T cells activity[271]. Several studies already described similar interactions on the KIR receptor of NK cells and the TCR from $CD8^+$ T cells with HLA-presented epitopes. Fadda *et al* showed that several HIV-1 common peptides variants, including the well-characterized TW10 epitope presented by HLA-B*57:01 and often associated with $CD8^+$ T cell mediated-immune escape, abrogated KIR3DL1 binding to HLA-B*57:01[173]. In addition, Hölzemer *et al* described that sequence polymorphisms in $T_{3gag303}V$, an immune-dominant epitope target by $CD8^+$ T cells and associated with $CD8^+$ T cells-mediated immune pressure, also affected KIR2DL3 binding to HLA-C*03:04 and $KIR2DL3^+$ NK cells function[139]. Interestingly, the "YIPLVGAPL" epitope identified in our study *(Chapter 1)* has already been described as an immune-dominant epitope for $CD8^+$ T cells when presented by HLA-A2[272]. It is therefore possible that early-emerging mutations resulting from $CD8^+$ T cells immune pressure could affect KIR^+ NK cell function, either in strengthening the binding to inhibitory KIRs or at the contrary, in abrogating binding to inhibitory KIRs and promote KIR^+ NK cell mediated lysis. Altogether, complexes interactions occurred between innate, adaptive immune pressure and viral evasion mechanisms, with the HLA-presented epitopes as central actors of these interplays.

In summary, our results provide evidence hinting at a novel HCV escape mechanism from $iKIR^+$ NK cell-mediated immune pressure, giving new insights into NK cell involvement in HCV disease outcome.

b. $KIR2DS1^+$ NK cells mediated-immune pressure?

For KIR2DS1, we identified a single HLA-C*06:02 presented peptide, "SRGPVHHLL", which modulated KIR2DS1 binding (*Chapter 2*). However, "SRGPVHHLL" is a synthetic peptide previously predicted to bind to HLA-C*06:02[241] and its sequence does not match any known viral or human epitope. In addition, we performed screening using over 568 HIV-1-derived 15-mer overlapping peptides. Five HIV-1 peptides stabilized HLA-C*06:02 expression, but strikingly, none of them allowed binding to KIR2DS1 *(Chapter 2*, *Figure III.3 b)*. As HLA class I-presented HIV-1 variants have already been described to bind to several iKIRs, including KIR2DL2 [172,174], KIR2DL3 [139] and

KIR3DL1 [173], it is remarkable that none of the tested HIV-1 peptides was able to engage binding of KIR2DS1. This might subject that HIV-1 has evolved specific mechanisms to avoid recognition of viral peptides by KIR2DS1$^+$ NK cells. KIR2DS1 is an activating NK cell receptor; a viral epitope binding to KIR2DS1 will consequently trigger KIR2DS1$^+$ NK cell activation and lysis of viral-infected cells. Thus, a putative escape mechanism for HIV-1 to avoid KIR2DS1$^+$ NK cell mediated-immune pressure would be to select variants abrogating KIR2DS1 binding.

In addition to HIV-1 peptides, we performed screenings of viral peptides derived from HCV, SIV, EBOLA and hCMV libraries *(Table 1, 4, 5 and 6, Appendix)*. These additional screenings were based on a) previous studies describing HLA-C-presented peptides from HCV or SIV affecting iKIR$^+$ NK cell function[260] *(Chapter 1)*, b) a genetic correlation between Ebola clinical outcome and KIR2DS1 expression[273] and c) the binding of primary KIR2DS1$^+$ NK cells to hCMV-infected Homozygous Fetal Foreskin Fibroblasts (HFFFs)[274]. Of note, we used an *in silico* pre-screening approach, based on algorithms software predicting peptides binding to HLA-I molecules, to restrict the number of HCV, EBOLA and hCMV peptides to test *in vitro.* [275]. Out of the pool of 745 peptides tested, 5 hCMV peptides were able to stabilize HLA-C*06:02 *(Figure III.3 a)* and were tested for KIR2DS1 binding *(Figure III.3 b)*. However, none of the peptides identified triggered a response in KIR2DS1ζ$^+$ Jurkat reporter cells.

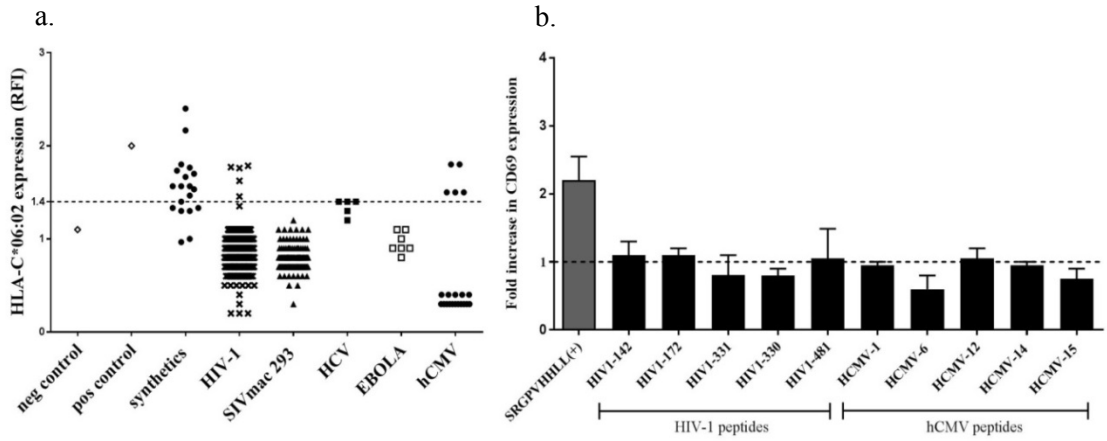

Fig III. 3 Impact of viral peptides presented by HLA-C*06:02 on KIR2DS1 binding.

*(a) Quantification of HLA-C*06:02 stabilization of 721.221-TAP1KO-C*06:02 pulsed with 19 different synthetic peptides, 568 HIV-1 clade B peptides, 125 SIVmac 293 GAG peptides, 5 HCV genotype 1 peptides (spanning core and NS3), 7 EBOLA peptides (Kikwit strain, GP protein) and 21 hCMV peptides (strain AD169, 65 kDa phosphoprotein). Peptides were added at a saturating concentration of 200 µM. MUM-2 derived peptide was used as positive control and the peptide "LLRHHNLIY" as a negative control (b) Bar graph showing fold increase in CD69 for KIR2DS1ζ$^+$ Jurkat cells, when co-incubated with 721.221-*

*TAP1KO-C*06:02 pulsed with different peptides (MdFI of the sample divided by the MdFI of KIR2DS1ζ+ Jurkat cells co-incubated with 721.221-TAP1KO-C*06:02 in the absence of peptide). Each bar represents median +/− interquartile range of 3 independent experiments.*

Very few studies have been able to describe peptide specificity for the activating NK cell receptor KIR2DS1. Stewart *et al* described one synthetic peptide, "QYDDAVYKL", presented by HLA-C*04:01 and binding to KIR2DS1, but the functional consequences of this interaction on KIR2DS1$^+$ NK cells was not demonstrated [155]. Additionally, peptide specificity for the interaction of HLA class I with aKIRs has been rarely demonstrated. Two HIV-1 peptides presented by HLA-B*57:01 were described to bind to KIR3DS1[276] and one vaccinia peptide presented by HLA-A*11 has been shown to impact binding to KIR2DS2[157]. Recently, it has been also reported that KIR2DS2 binds to HLA-C*01:02-presented peptides derived from conserved regions of flaviviral superfamily 2 RNA helicase[277]. Similarly to the study of Stewart *et al*, no functional consequences for recognition by primary aKIR$^+$ NK cells were assessed. Finally, we demonstrated that primary KIR2DS1$^+$ NK cell clones are activated by 721.221.HLA-C*06:02 *(Chapter 2)* and Stewart *et al*, demonstrated binding to 721.221.HLA-C*04:01[155], both EBV transformed B cell lines *(Table III.1)*. Recently, activation of primary KIR2DS1$^+$ NK cells was demonstrated against HFFFs HLA-C2$^+$, but only when infected with specific clones of a clinical strain of human cytomegalovirus (hCMV, B6, T40/E)[274]. Altogether, we establish peptide-dependent activation of KIR2DS1$^+$ NK cells, but the lack of viral peptides identified suggests that others mechanisms are required to fully explain the role of KIR2DS1$^+$ NK cells during viral infections. There is a possibility that specific viral mechanisms might have evolved to avoid recognition of viral peptides by activating NK cells receptors, however, two alternative hypotheses will be discussed in the next chapters: (1) the primary function of KIR2DS1$^+$ NK cells is not focused on the defense against viral infections and its main role may be confined to tissue remodeling. (2) Additional factors modulating HLA-C-presented peptides are required to bind KIR2DS1 and influence KIR2DS1$^+$ NK cells function.

III. Differential function of KIR2DS1$^+$ NK cells

a.iKIRs and aKIRs have different functions.

The precise ligand(s) and functions of KIR2DS1 remain elusive. On the contrary, its inhibitory counterpart, KIR2DL1, is better understood and the crystal structure of KIR2DL1 in complex with its ligand, HLA-C*04:01, has been resolved[154]. KIR2DS1 and KIR2DL1 share a high degree of sequence homology (97% in their extracellular domain)[187]. Moreover, inhibitory KIRs have often been described as ancestral, the activating receptors having evolved from them by mutation suggesting that KIR2DS1 might have evolved from KIR2DL1[278]. Therefore, KIR2DS1 is often analyzed in the context of KIR2DL1. In an effort to better understand KIR2DS1$^+$ NK cell function, we performed a systematic comparison with KIR2DL1$^+$ NK cells.

Our study showed that KIR2DL1 and KIR2DS1 shared similar binding specificities to HLA-C2 molecules, consistent with previous studies[155,233,235] and shared the peptide-specific recognition of HLA-C*06:02-presented peptide "SRGPVHHLL" *(Chapter 2)*. Interestingly, KIR2DL1 showed a stronger affinity than KIR2DS1 for HLA-C*06:02 and "SRGPVHHLL" in functional assays *(Chapter 2)*. These results are consistent with previous studies showing that inhibitory receptors have a stronger binding affinity than their corresponding activating NK cell receptors[235,279]. As the majority of circulating peripheral blood NK cells (pNK cells) co-express KIR2DS1 and KIR2DL1[280], only a minority of KIR2DS1$^+$ pNK cells can be efficiently activated by HLA-C2-presented peptides. In addition, KIR2DS1$^+$ pNK cells have been described to be hyporesponsive in donors expressing HLA-C2[281], indicating education of aKIRs via their MHC ligand to turn down their responsiveness in order to prevent autoimmunity. Traditionally, NK cell education can be defined as a mechanism in which NK cells acquire full maturation and functional competence after recognition of self-HLA molecules by inhibitory receptors, specifically iKIRs[282]. The precise mechanisms underlying NK cell education remain however unclear and the education of NK cells through aKIRs complicate the existing models. Interestingly, the hyporesponsiveness of KIR2DS1$^+$ pNK cells in individuals expressing HLA-C2 is only limited to target cell recognition, as KIR2DS1$^+$ pNK cells strongly respond to exogenous stimulation with cytokines (IL-12 and IL-15) by secreting IFN-γ[281]. Therefore, it is possible that the role of KIR2DS1$^+$ NK cells rather focuses on

cytokines production and KIR2DS1$^+$ NK cells might primarily act as immunoregulatory cells.

Altogether, our data, in line with previous studies, suggest that the activating NK cell receptor KIR2DS1 mediates limited effector function in the peripheral blood due to a stronger binding affinity of its inhibitory counterpart KIR2DL1, which might have evolved to limit the risks of autoimmune diseases.

b. Tissue specific role of KIR2DS1 in pregnancy

KIR2DS1 has often been described to play an important role in pregnancy, indicating a possible tissue-specific role for KIR2DS1$^+$ NK cells subset[283]. If the fetus encodes for HLA-C2, mothers carrying the KIR2DS1 allele have an increased pregnancy success[217]. Several studies suggested that the interactions of maternal uterine KIR2DS1$^+$ NK (uNK) cells with the fetal trophoblasts expressing HLA-C2 impacts fetus birthweight and reproductive success[216]. Functional studies described the uNK cells subset as distinct from pNK cells in terms of phenotype and function[78]. uNK cells are mainly CD56^{bright}CD16$^-$, poorly cytotoxic and preferentially produce cytokines [78]. Additionally, uNK cell subsets have been shown to express higher frequencies of KIR2DS1 and KIR2DL1 in comparison to pNK cells from the same donor[210,220,284] and to produce higher quantities of cytokines even when KIR2DL1 is co-expressed on the cell[284]. Indeed, activated KIR2DS1$^+$ uNK cells express cytokines, in particular the Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), which attract trophoblast cells and induce a remodeling of maternal arteries for a better supply of oxygen and nutrients to the fetus[210] Altogether, KIR2DS1$^+$ NK cells could have a particular function in pregnancy and reproductive success as cytokine producers. Moreover, genetically, KIR2DS1 allele is part of the KIR B haplotype which is often associated, together with the HLA-C2 molecule, with a better reproductive success and with fetus of larger size[231]. On the contrary, KIR haplotype A and HLA-C1 molecules are linked with better resistance to pathogens[285]. Within the human Caucasian population, these two haplotypes are both maintained at approximately 50% each, independently of sex[286], suggesting that these haplotypes are subject to natural selection depending on pathogen load and reproductive pressure. Nonetheless, the tissue-specific function of KIR2DS1$^+$ NK cell is restricted to uNK cells and the precise role of KIR2DS1$^+$ pNK cells remains unclear.

c. Role of KIR2DS1 in autoimmune disease

KIR2DS1 is also described to be associated with increased susceptibility to autoimmune diseases[159] depending on the HLA-C allotype. It is associated with risk of Psoriasis Vulgaris in the presence of HLA-C*06:02[208,221,222], Psoriasis Arthritis in the absence of HLA-C2 alleles[209,223,224], Scleroderma[225,226], Systemic Lupus Erythematosus[225,227] and Ankylosing Spondylitis in association with HLA-C2[228] *(Table I, 3)*. These results are based on genetic studies looking both at *KIR* and *HLA* genes and need further functional investigations. However, these results indicate a potential break of tolerance of pKIR2DS1$^+$ NK cells, rendering them responsive against target cells and able to mediate autoimmune reactions and this, even in the absence of HLA-C2 ligands. Therefore, KIR2DS1$^+$ NK cells may be able to recognize other ligands in the absence of HLA-C2.

IV. Additional factors potentially involved in KIR2DS1 binding

An alternative, but not mutually exclusive, hypothesis explaining the lack of HLA-C2-restriceted viral peptides identified to bind to KIR2DS1 and the stronger binding affinity of KIR2DL1 for HLA-C2 than KIR2DS1 could be the existence of an additional non HLA-C2 high-affinity ligand for KIR2DS1. In addition to our study, several groups have investigated the binding specificities of KIR2DS1[155,235,274,287–290] *(Table III.1 and 2)*.

Table III.1. Cell lines described to bind KIR2DS1*.

Cells line binding to KIR2DS1	Type of cells	System and reference
721.221.HLA-C*04:01/C*06:02 (HLA-C2) +/- synthetic peptide	EBV-transformed HLA-class-I deficient B cells lines transfected with respective HLA allele.	KIR2DS1ζ^+ Jurkat reporter cells, pKIR2DS1$^+$ NK clones[291]. KIR2DS1 chimeric soluble receptor, KIR2DS1 NK clone.[235] KIR2DS1 tetramer.[155] note: does not bind with KIR2DS1$^+$ 2B4 reporter cells[274].
BL30-B95 (HLA-C2/C1)	EBV-infected Burkitt lymphoma cancer cells line	KIR2DS1 tetramer[155]
HLA-C2 BLCL	B lympho-blastoid cells lines	Polyclonal NK cells from KIR2DS1$^+$ HLA-C1/C1 donor Primary KIR2DS1$^+$ NK cell clones[290,292].
HLA-C2 leukemia cells	Hematopoietic stem cells, CD34+ from pediatric donors with hematologic malignancies	KIR2DS1 soluble receptors, polyclonal KIR2DS1$^+$ primary NK cells, KIR2DS1$^+$ NK cell clones[288]
HLA-C2/C2 and C2/C1 T cell blast and mDCs	Primary myelomocitic dendritic cells and T cells from heathy HLA-C2 donors	KIR2DS1$^+$ NK cell clones[289]

hCMV infected HFFF (specific clone B6, T40/E) (HLA-C2)	Human Foreskin Fibroblast neonatal infected with HCMV, strain T40/E	KIR2DS1$^+$ 2B4 reporter cells, primary KIR2DS1$^+$ NK cells[274]

**: The studies presented were limited to peripheral NK cells. HLA class I type of the cell lines is indicated when described.*

KIR2DS1 has been described to bind to several cell lines, mostly to B-cell lines *(Table III. 1)*. Of note, the cell lines identified share common features: they are derived from human sources, express HLA-C2 molecules and were either EBV-, HCMV-infected or malignantly transformed. We performed anti HLA-C antibody blocking experiments on 721.221.HLA-C*06:02 (data not shown) and 721.221.HLA-C*06:02 presenting the peptide "SRGPVHHLL". Blocking of HLA-C resulted in abrogation of KIR2DS1ζ^+ Jurkat reporter cell activation, confirming that HLA-C2 is necessary for KIR2DS1 binding *(Chapter 2)*. Additionally, 221.TAP1KO.HLA-C*06:02 showed weaker activation of KIR2DS1ζ^+ Jurkat reporter cells and weaker activation of KIR2DS1$^+$ NK cell clones *(Chapter 2)*. Abrogation of KIR2DS1 binding has also been shown by Von Ploeg *et al*, by performing knocking out the β2M gene involved in the formation of the HLA class I complex[274]. Altogether, these results confirmed that HLA-C2 molecules are required for KIR2DS1 binding. However, these HLA-C2/peptide complexes alone were not sufficient to induce primary KIR2DS1$^+$ NK cells activation *(Table III. 2)*.

Table III.2. Cell lines described to not bind KIR2DS1*.

Cells line not binding to KIR2DS1		**system**
721.221	EBV-transformed HLA-class-I deficient B cells line.	KIR2DS1ζ^+ Jurkat reporter cells[291]. KIR2DS1$^+$ BWN3G reporter cells[287]. soluble chimeric KIR2DS1 receptor[235].
721.221.HLA-C*03:04, HLA-C*07:02 (HLA-C1)	EBV-transformed HLA-class-I deficient B cells line transfected with indicated HLA allele	KIR2DS1ζ^+ Jurkat reporter cells.[291] KIR2DS1$^+$ BWN3G reporter cells.[287] KIR2DS1$^+$ 2B4 reporter cells.[274] Soluble chimeric KIR2DS1 receptor.[235]
221.HLA-A*11:02, A*23:01	EBV-transformed HLA-class-I deficient B cells line transfected with indicated HLA allele	KIR2DS1$^+$ 2B4 reporter cells[274].
221.HLA-Bw4, Bw6	EBV-transformed HLA-class-I deficient B cells line transfected with indicated HLA allele	KIR2DS1$^+$ 2B4 reporter cells[274]. soluble chimeric KIR2DS1 receptor[235].
CHO/CHO-HLA-C1/CHO-HLA-C2	Chinese hamster ovary cells line alone or stably transfected with HLA-C*01:02/C*03:04/C*07:02/C*08:02	KIR2DS1$^+$ 2B4 reporter cells[274].

	(HLA-C1) or HLA-C*06:02 (HLA-C2)	
SK-BR-3 (HLA-C1)	Human adenocarcinoma cell line derived from mammary gland/breast.	KIR2DS1$^+$ BWN3G reporter cells[287].
OVCAR-3 (HLA-C1)	Human adenocarcinoma cells line derived from ovary	KIR2DS1$^+$ BWN3G reporter cells[287].
T47D (HLA-C1)	Human ductal carcinoma cells line derived from mammary gland	KIR2DS1$^+$ BWN3G reporter cells[287].
WN35 (HLA-C1/C2)	Human human melanoma cells line	KIR2DS1$^+$ BWN3G reporter cells[287].
PC-3 (HLA-C1/C2)	Human prostate cancer cells line	KIR2DS1$^+$ BWN3G reporter cells[287].
DU145 (HLA-C2)	Human prostate cancer cells line	KIR2DS1$^+$ BWN3G reporter cells[287].
HeLa (HLA-C1)	Human adenocarcinoma cells line from cervix	KIR2DS1$^+$ 2B4 reporter cells[274].
MelJuSo (HLA-C1)	Human meloma cells line	KIR2DS1+ 2B4 reporter cells[274].
Ca Ski	Human epidermoid carcinoma cells line from cervix	KIR2DS1+ 2B4 reporter cells[274].
JEG-3	Human placenta choriocarnoma cells line	KIR2DS1+ 2B4 reporter cells[274].
BL-30	Burkitt lymphoma cells line	KIR2DS1 tetramer[155].
MRC-5 infected with HSV-1, HSV-2, HCMV	Fibroblast cells line infected with HSV-1, HSV-2 or HCMV	KIR2DS1 tetramer[155].

**: The studies presented were limited to peripheral NK cells. HLA class I type of the cell lines is indicated when described.*

Indeed Chinese Ovary Hamster (CHO) cell lines stably transfected with HLA-C*06:02 as well as several malignant cell lines expressing HLA-C2 (DU145, WN3S, PC3) did not activate KIR2DS1$^+$ BWN3G reporter cells[287]. Altogether, these results indicated that an additional factor may be required to activate KIR2DS1$^+$ NK cells or allow for KIR2DS1-mediated recognition of HLA-C2:peptide complexes.

a. HLA-C2-independent ligand

First, we discuss the question if an additional HLA-C2 independent ligand with a stronger affinity for KIR2DS1 than HLA-C2/peptide complexes exists. KIR2DS1 could potentially recognize pathogen-encoded, -induced, or -altered ligands. In this case, the specificity of KIR2DS1 for HLA-C2 molecules would reflect cross- reactivity with KIR2DL1.

Several non-classical HLA class I ligands have been described for activating NK cell receptors. Thiruchelvam-Kyle *et al*. did report an HLA class I-independent ligand expressed on cancerous cells for KIR2DS2, yet did not identify the exact ligand. Another aKIR, KIR2DS4, has been suggested to bind to an unidentified protein expressed on melanoma-derived tumor cells, independently of HLA class I[293]. The Murine CMV

(MCMV) protein m157 encoded by infected cells has been reported to directly bind to the activating murine NK cell receptor Ly49H[294], suggesting that pathogen-encoded ligands can activate NK cells, but no viral ligand has been described for human activating KIRs. Finally, recent studies established that KIR3DS1 binds to the non-classical HLA class I HLA-F molecules. HLA-F exists in two conformations, as a peptide-loaded form and as an open conformer, of which only HLA-F Open Conformers (OC) are recognized by KIR3DS1 (open conformers are corresponding to a HLA molecule lacking β_2M and the peptide)[158,295]. Additionally, here we analyzed KIR2DS1-binding to the non-classical HLA class I molecules HLA-E –F and –G, both as heterotrimeric complexes and OCs, but did not detect any KIR2DS1 binding to these molecules *(Fig III. 4)*. Therefore, the mechanism employed by KIR2DS1 in recognizing malignant cells is not directly mimicking the one employed by KIR3DS1, suggesting a different ligand or process for KIR2DS1.

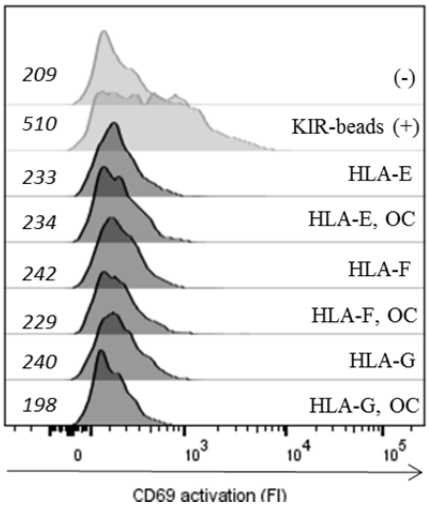

Fig III. 4 Binding of KIR2DS1ζ$^+$ Jurkat reporter cell lines to beads coated with non-classical HLA class I molecules. *Results are shown as representative flow cytometry histogram showing Fluorescence Intensity (FI) for CD69 activation for KIR2DS1ζ$^+$ Jurkat reporter cell lines. Italic numbers indicate the median of CD69 FI. Biotinylated monomers of HLA-E+β2m+VMAPRTLVL, HLA-F+β2m, and HLA-G+β2m+KGPPAALTL were kindly provided by Dr. Wilfredo Garcia-Beltran (Ragon Institute of MGH, MIT and Harvard, Cambridge, US) and loaded onto streptavidin-coated beads (Life Technologies). Open conformers were generated by acid pulsing as described in[158]. Beads were incubated with KIR2DS1ζ$^+$ Jurkat reporter cell lines at a ratio 1:50 for 3h at 37°C, washed with PBS 2% FBS, resuspended in 4% PFA and analyzed by flow cytometry (BD LSR Fortessa).*

Altogether, despite our systematic screening of KIR2DS1 binding specificity to various classical and non-classical HLA class I molecules and the screening of various cell lines

performed by different groups[274,287], no pathogen-encoded, -induced, or -altered ligands have been identified so far. Taken together, combined data indicates that KIR2DS1-binding occurs in the presence of HLA-C2 molecules with no indication of an independent high-affinity ligand to date. Thus, we hypothesize that a modified form of HLA-C2 might mediate KIR2DS1$^+$ NK cell activation.

b. Modified form of HLA-C2

As mentioned above, all the cell lines described to bind to KIR2DS1 were either EBV-, CMV- or malignantly transformed. Therefore, we hypothesize that an altered form of HLA-C2 might be required to induce KIR2DS1$^+$ NK cell activation. KIRs as well as HLA-C ligands gather in nanoclusters on the cellular membrane and form immune synapses necessary for phosphorylation signaling in NK cells[296]. KIR2DS1 molecules were shown to assemble in larger clusters than KIR2DL1 at the cell surface[297], so potentially a modified form of HLA-C2 may increase the clustering of KIR2DS1, leading to NK cell activation.

Several possibilities exist: First, viruses may alter posttranslational modification of HLA class I/peptides complexes, for example alteration of glycosylation patterns, and thus increase binding affinity to KIR2DS1. HLA class I alterations by glycosylation have been shown to impact KIR3DL1 interaction with HLA-B*57:01[247]. Moreover, the use of tunicamycin, an inhibitor of the first step of N-glycosylation, significantly reduced KIR3DL1-Fc binding and decreased activity of KIR3DL1$^+$ NK cell clones[247]. Second, HLA-C2 molecules might form dimers either with other HLA-C2 (homodimers) or with other HLA class I molecules (heterodimers). Free HLA class I H chains can be expressed as dimers[298] and may form heterodimers, as previously reported for HLA-F dimers with open conformers of HLA class I[299]. Lastly, HLA-C free heavy chains have been described to associate with the HIV-1 protein Env at the cell surface of infected cells[300], suggesting that viral ligands also can form dimers with HLA-C2 molecules.

Altogether HLA-C2 complexes efficiently induced KIR2DS1 binding. Previously, this has been shown using different KIR2DS1 reporter systems[274,287] *(Chapter 2)* and soluble KIR2DS1 molecules[155], but these complexes were generally not sufficient to activate primary KIR2DS1$^+$ NK cells. In our study, we showed for the first time that HLA/peptides complexes can activate primary KIR2DS1$^+$ NK cell clones *(Chapter 2)* but the lack of viral peptide identified suggest that another factor associating to or modulating

HLA-C2 might be required for KIR2DS1^{+} NK cells function. The use of new tools such as genome-wide CRISPR-based screen[301] applied on cell lines described to bind to KIR2DS1 could uncover genes necessary for KIR2DS1 interaction to its target cell lines. Moreover, resolving the crystal structure of KIR2DS1 in complex with HLA-C2-presented peptides may help to better understand the mechanism underlying KIR2DS1 binding.

CONCLUSION

This thesis studied the impact of HLA class I-presented peptides on KIR^+ NK cells in the context of HIV-1 and HCV disease. We aimed to (1) Identify HCV peptides presented by HLA-C*03:04, which influence KIR2DL3 binding to HLA-C*03:04 and $KIR2DL3^+$ NK cell function and (2) Identify peptides modulating the KIR2DS1/HLA-C*06:02 interaction and study their impact on the function of $KIR2DS1^+$ NK cells. Our findings can be summarized as follows:

Chapter 1

- Identification of a single HCV peptide "YIPLVGAPL", derived from the core protein of HCV genotype 1, stabilizing HLA-C*03:04 and binding to KIR2DL3.
- The identified HLA-C*03:04 peptide "YIPLVGAPL" significantly inhibited primary $KIR2DL3^+$ NK cell degranulation
- Several naturally occurring sequence variations of "YIPLVGAPL" in other HCV genotypes showed a reduced capacity to bind to KIR2DL3 and to inhibit $KIR2DL3^+$ NK cell function.

Taken together, these results indicated that HLA class I presentation of HCV-derived peptides impact $iKIR^+$ NK cell functions and provide first insights into a novel mechanism by which HCV may be able to evade NK cell-mediated immune pressure through the selection of sequence variations.

Chapter 2

- Identification of the synthetic peptide "SRGPVHHLL" stabilizing HLA-C*06:02 and binding to KIR2DS1.
- The HLA-C*06:02 peptide identified "SRGPVHHLL" induced activation of primary $KIR2DS1^+$ NK cell clones.
- No naturally occurring virus-derived peptides binding to KIR2DS1 were identified.

Taken together, these results confirmed the peptide-dependent binding of KIR2DS1 to HLA-C2 molecules, but the lack of a viral peptide identified suggests that additional

factors might be necessary to induce functional activity of KIR2DS1$^+$ NK cells in the context of viral infections.

Several studies showed that KIR/HLA associations are linked with HIV-1 and HCV disease outcome. Therefore a better understanding of the impact of HLA class I-presented peptides on KIR$^+$ NK cells will help uncover new insights into the mechanisms involved in HCV and HIV-1 pathogenesis and might provide new perspectives for NK cell immunotherapy.

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Indices

Figures

Tables

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Thank you to all the anonymous blood donors which gave me the principal material to perform my experiments and special thanks for one of them, from which I could create wonderful primary KIR2DS1^{+} NK cell clones.

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Finally I would not there without the help of my family and I want to dedicate them this thesis. To my mother, my father and my sister Océane, I want to thank you for never giving up in me, even when I was, and for constant and loving support.

Participation in publications

I. Sequence variations in HCV core-derived epitopes alter binding of KIR2DL3 to HLA-C∗03:04 and modulate NK cell function.

I was involved in the realization of the experiments. I also participated in the discussion of the data and comments on the manuscripts.

II. Peptide-specific engagement of the activating NK cell receptor KIR2DS1.

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

Curriculum Vitae

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Professional experience

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Department of Viral Immunology, Prof. Dr. Marcus Altfeld
Study of the effect of HLA class I-peptides on KIR+ NK cell function in the context of viral infections (HIV-1 and HCV)

February-August 2013: **Master thesis at the Ragon Institute of MGH, MIT and Harvard, Boston, United State**
Department of Dr. Galit Alter, innate immunity
Analysis of SNP associated biomarkers in infectious and autoimmune diseases

July-August 2012: **Voluntary internship at ISTEM (stem cell institute for the treatment and study of monogenic diseases).** Evry genopole, France
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June-July 2011: **Voluntary internship at Urolead, INSERM start-up of the medical University of Strasbourg.** Kidney cancerology section, France
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Education

2010-2013: **Biotechnologies Engineer diploma in the European School of Biotechnologies in Strasbourg (ESBS). Strasbourg; France**

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Cell biology: Flow cytometry, Fluorescence-activated cell sorting (FACS), PBMC isolation, T cell and NK cell isolation, NK cells degranulation and killing assays, cell culture, cell transfection, lentiviral transduction, binding assays with reporter cell lines.
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Publications

1. **Anais Chapel**, Wilfredo F. Garcia-Beltran, Angelique Hölzemer, Maja Ziegler, Sebastian Lunemann, Gloria Martrus, Marcus Altfeld: Peptide-specific engagement of the activating NK cell receptor KIR2DS1. Scientific Reports 05/2017; 7(1), DOI:10.1038/s41598-017-02449-x

2. Claudia Beisel, Susanne Ziegler, Glòria Martrus Zapater, **Anaïs Chapel**, Morgane Griesbeck, Heike Hildebrandt, Ansgar W. Lohse, Marcus Altfeld: TLR7-mediated activation of XBP1 correlates with the IFN-α production in humans. Cytokine 04/2017, DOI:10.1016/j.cyto.2017.04.006

3. Sebastian Lunemann, Gloria Martrus, Angelique Hölzemer, **Anais Chapel**, Maja Ziegler, Christian Körner, Wilfredo Garcia Beltran, Mary Carrington, Heiner Wedemeyer, Marcus Altfeld: Sequence variations in HCV core-derived epitopes alter binding of KIR2DL3 to HLA-C∗03:04 and modulate NK cell function. Journal of Hepatology 04/2016, DOI:10.1016/j.jhep.2016.03.016

4. B M Gunn, J R Schneider, Maryam Shansab, Arangassery Rosemary Bastian, K M Fahrbach, A D Smith, A E Mahan, M M Karim, A F Licht, Ivan Zvonar, Jacquelynn Tedesco, M R Anderson, **Anais Chapel**, T J Suscovich, D C Malaspina, Hendrik Streeck, Bruce D. Walker, Arthur Kim, Georg Lauer, Marcus Altfeld, Shiv Pillai, Igal Szleifer, Neil L. Kelleher, Patrick F. Kiser, Thomas J. Hope, Galit Alter: Enhanced binding of antibodies generated during chronic HIV infection to mucus component MUC16. Mucosal Immunology 03/2016; 9(6), DOI:10.1038/mi.2016.8

International Talks and Posters

Talk: *KIR2DS1 reporter cell assay to identify HIV-1 peptide specific recognition of HLA-C*06:02*
The KIR polymorphism workshop, September 10-12, 2015, Winchester, UK

Posters: *Identification of HLA/peptide complexes binding to the activating NK cell receptor KIR2DS1*
16th Annual Meeting of the Society for Natural Immunity, October 2-5, 2016, Taormina, Sicily

*Optimization of KIR2DS1 reporter cell assay to identify HIV-1 peptide specific recognition of HLA-C*06:02 receptor*
5th European AIDS Conference, October 21-24, 2015, Barcelona, Spain
Natural Killer Symposium, October 7-9, 2015, Göttingen, Germany

Extracurricular Activities

Associative life: Organization of the HPI PhD Student and Postdoc Retreat 24-25.11.2016, Hamburg, Germany
Helper at the 6th European Congress of Virology, October 19-22.2016, Hamburg, Germany.
Active member of the Student Office of ESBS: responsible for external relations, active member of the POLY (association organizing events for all engineering schools of Strasbourg): responsible for the funding of sponsors
Animator for summer camps –BAFA diploma (qualified child worker/play leader) (Summer 2008-2009)
Sports: kickboxing (three years), judo (ten years, competition level), oriental dancing (three years)
Hobbies: literature, travels (Japan, Canada, Egypt, Great Britain, Ireland…)

Bestätigung der Korrektheit der englischen Sprache

Hiermit bestätige ich, Christopher Thomas Ford geboren am 30. Juli 1990 in Edinburgh, Scotland (UK), dass die Dissertation von Anais Chapel mit dem Titel "Effect of HLA class I-peptides on KIR+ NK cell function in the context of viral infections" in einem korrekten Englisch verfasst wurde.

HH 12/07/17. _____

Datum, Ort Unterschrift

Eidesstattliche Versicherung

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

13/07/2017 Hamburg

Amos chapel

Appendix

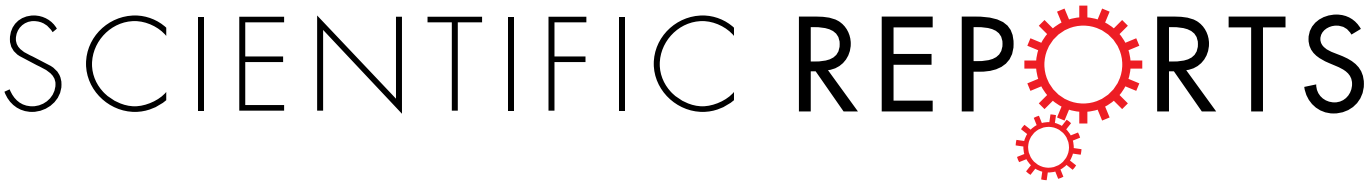
Papers:

Chapter I: *Sequence variations in HCV core-derived epitopes alter binding of KIR2DL3 to HLA-C∗03:04 and modulate NK cell function*.

Chapter II: *Peptide-specific engagement of the activating NK cell receptor KIR2DS1*.

Other:

Chapter 1



OPEN

Peptide-specific engagement of the activating NK cell receptor KIR2DS1

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Anaïs Chapel[1], Wilfredo F. Garcia-Beltran[3], Angelique Hölzemer[2], Maja Ziegler[1], Sebastian Lunemann[1], Gloria Martrus[1] & Marcus Altfeld[1]

The activating NK cell receptor KIR2DS1 has been shown to be involved in many disorders including autoimmune diseases, malignancies and pregnancy outcomes. However, the precise ligands and functions of this receptor remain unclear. We aimed to gain a better understanding of the factors involved in the binding of KIR2DS1 and its inhibitory counterpart KIR2DL1 to HLA class I molecules, and the consequences for KIR2DS1+ NK-cell function. A systematic screen that assessed binding to 97 HLA-I proteins confirmed that KIR2DS1-binding was narrowly restricted to HLA-C group 2 complexes, while KIR2DL1 showed a broader binding specificity. Using KIR2DS1ζ$^+$ Jurkat reporter-cells and peptide-pulsed 721.221.TAP1KO-HLA-C*06:02 cells, we identified the synthetic peptide SRGPVHHLL presented by HLA-C*06:02 that strongly engaged KIR2DS1- and KIR2DL1-binding. Functional analysis showed that this HLA-C*06:02-presented peptide can furthermore activate primary KIR2DS1(+) NK cell clones. Thus, we demonstrated peptide-dependent binding of the activating NK cell receptor KIR2DS1, providing new insights into the underlying mechanisms involved in KIR2DS1-related disorders.

Natural killer (NK) cells play a pivotal role in containing viral replication in early stages of infection and in shaping the subsequent adaptive immune response[1]. NK cells are able to recognize and kill abnormal cells thought multiple receptors that distinguish normal host molecules, stress-induced ligands, and pathogen-associated motifs[2]. These receptors are either activating or inhibitory and constitute a fine balance of signals which tightly controls NK cell function. One of the major families of NK cell receptors, the Killer Immunoglobulin Receptors (KIRs), has been shown to impact the outcome of various diseases, in particular in association with their Human Leukocyte Antigen (HLA) class-I ligands[2–4].

KIR family receptors are encoded by polymorphic and highly homologous genes located on human chromosome 19q13.4 within the leukocyte receptor complex (LRC)[5]. Although KIRs are characterized by an extensive number of haplotypes, they all share a similar molecular structure consisting of a type 1 transmembrane glycoprotein with ectodomains comprising either two (KIR2D) or three (KIR3D) immunoglobulin-like domains[3]. The length of the cytoplasmic tail determines whether a respective KIR is inhibitory or activating: a long cytoplasmic tail characterizes inhibitory KIRs (KIR-L) whereas a short cytoplasmic tail characterizes activating KIRs (KIR-S). Most KIRs interact with specific allotypes of HLA class I ligands[5]. In general, receptors of the KIR3D group engage HLA-A and HLA-B while KIR2D receptors interact with HLA-C molecules. HLA-C ligands can be subdivided into two groups: HLA-C group 1 (HLA-C1), characterized by an asparagine in position 80, binds to KIR2DL2 and KIR2DL3 molecules and HLA-C group 2 (HLA-C2), characterized by a lysine in position 80, preferentially binds to KIR2DL1 molecules[5].

A growing number of studies have identified associations between the presence of the activating KIR2DS1 receptor and susceptibility to autoimmune diseases[6–8], reproductive success[9,10], control of viral infections[11,12] and malignancy in cancer[13–15]. However, the precise ligands for KIR2DS1, and their consequences for KIR2DS1+ NK-cell function, are not well characterized. KIR2DS1 and KIR2DL1 are alleles of the same single locus and share a high degree of sequence homology in their extracellular domain[16,17]. KIR2DS1 is distinguished by having two additional residues in the transmembrane region (Lysine 233 and Threonine 237), which interact with DAP12, an adaptor protein containing immunoreceptor tyrosine-based activation motif (ITAM)[18]. For this reason, KIR2DS1

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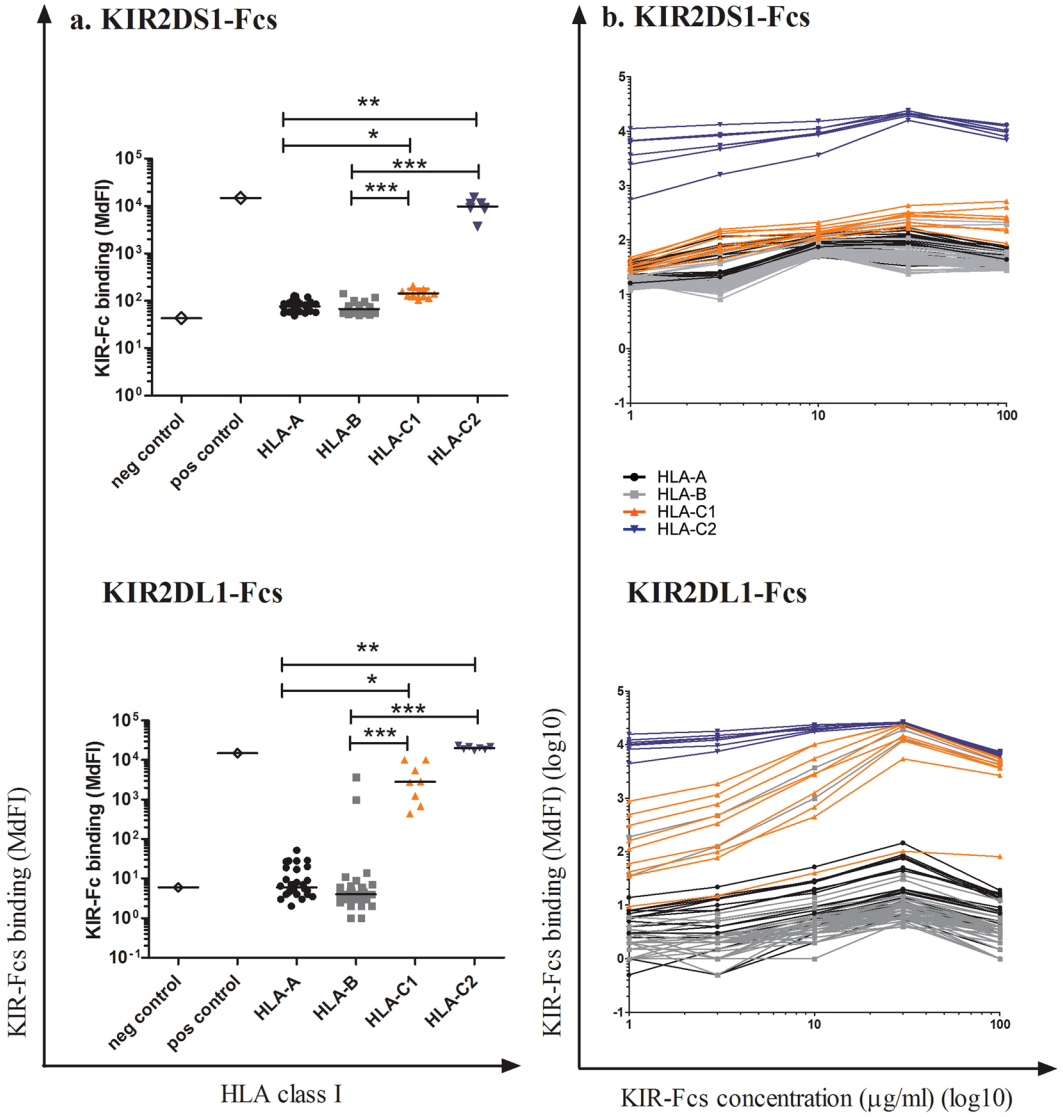

Figure 1. Systematic screening of KIR2DS1 and KIR2DL1 binding specificities. (**a**) KIR-Fc binding to classical HLA-I coated beads (One Lambda). KIR-Fcs were used at a concentration of 10 µg/ml. Median for each HLA-I subclasses are shown (**b**) Titration of KIR-Fcs binding to classical HLA-I coated beads (One Lambda).

and KIR2DL1 are generally considered as counterparts sharing the same ligand-specificity for HLA-C2 allotypes[16]. Nevertheless, crystal structure analysis of KIR2DL1 bound to HLA-C*04:01 has demonstrated that binding of KIR2DL1 is not only determined by the motifs located on the heavy chain of the HLA class I molecule but also by the sequence of the peptide presented by HLA class I[19–21]. Much less is known about the mechanisms that regulate binding of KIR2DS1 to HLA-C2[17]. It has been shown that peptides presented by the HLA-C2 molecule HLA-C*04:01 can also modulate KIR2DS1-binding[22,23], but the functional consequences of these interactions remain unclear. Here, we demonstrate that KIR2DS1-binding is narrowly restricted to HLA-C2 ligands while KIR2DL1 exhibited a broader HLA-C ligand specificity. Furthermore, specific HLA-C*06:02-presented peptides can modulate KIR2DS1-binding and activation of primary KIR2DS1+ NK cell clones.

Results

KIR2DS1 narrowly binds to HLA-C2 molecules, while KIR2DL1 has broader binding specificity for HLA class I molecules. A multiplex bead-based binding assay (One Lambda) consisting of 97 different beads coated with the most common allotypes of HLA-A, B, C was used to systematically assess HLA class I complex-binding to KIR2DS1- and KIR2DL1-fusion constructs (Fcs) as previously described[16,46] (Fig. 1). KIR2DS1 binding was exclusively restricted to HLA-C2 complexes tested (HLA-C*02:02,*04:01,*05:01,*06:02,*15:02,*17:01,*18:02), while its inhibitory counterpart KIR2DL1 showed a broader specificity (Fig. 1a) also binding to eight HLA-C1 complexes (HLA-C*01:02,*03:02,*03:03,*03:04,*07:02,*08:01,*12:03,*16:01) as well as two HLA-B complexes (HLA-B*46:01 and HLA-B*73:01). To further study the avidity of binding of HLA-I molecules to KIR2DS1 and KIR2DL1,

a titration of KIR-Fcs was performed ranging from 1 to 100 μg/ml (Fig. 1b). KIR2DS1 had a high avidity for HLA-C2 complexes and reached a saturation point at 30 μg/ml. However, analysis of KIR2DL1-Fcs titrations showed two distinct binding groups with different avidity: KIR2DL1 showed high avidity binding to HLA-C2 complexes with a saturation point at 30 μg/ml, but weaker binding to the eight additional HLA-C1 and the two HLA-B complexes. The results confirmed a model in which KIR2DS1 and KIR2DL1 share the same ligand specificity for HLA-C2 complexes, but KIR2DL1 can also bind to additional HLA-C1 and HLA-B complexes with weaker affinity. Interestingly KIR2DL1-binding to the HLA-C1 complexes HLA-C*01:02, HLA-C*03:02, HLA-C*03:03, HLA-C*03:04, HLA-C*07:02, HLA-C*08:01, HLA-C*2:03 and HLA-C*16:01 was not described before, while the receptor encoded by the KIR2DL1*022 allele has already been reported to bind to HLA-B*46:01 and HLA-B*73:01[24].

KIR2DS1ζ$^+$ and KIR2DL1ζ$^+$ Jurkat reporter cells are activated by 721.221 cells expressing HLA-C2 molecules. To determine the functional consequences of the above observed binding between KIR2D and HLA-C molecules, KIR2DS1ζ$^+$ and KIR2DL1ζ$^+$ -expressing Jurkat reporter cells were incubated with 721.221 cells stably transduced with specific HLA-C1 ligands (HLA-C*03:04, HLA-C*07:02) or HLA-C2 ligands (HLA-C*04:01, HLA-C*06:02) (Fig. 2a and b). HLA-devoid 721.221 cells were used as negative controls. Ligand engagement of KIR2DS1ζ$^+$- or KIR2DL1ζ$^+$-expressing Jurkat cells resulted in an activating signal through CD3ζ that triggered CD69 expression. Beads coupled with KIR2DL1/S1 (HPMA4) antibodies were used as a positive control. KIR2DS1ζ$^+$ Jurkat cells were significantly activated by 721.221. cells expressing HLA-C*04:01 or HLA-C*06:01, while 721.221- cells expressing HLA-C*03:04 and HLA-C*07:02 did not activate KIR2DS1ζ$^+$ Jurkat cells. KIR2DL1ζ$^+$ Jurkat cells were activated by 721.221- cells expressing HLA-C*04:01 and HLA-C*06:02 to a higher degree, while 721.221- cells expressing HLA-C*03:04 and HLA-C*07:02 only weakly activated KIR2DL1ζ$^+$ Jurkat cells. This results confirmed the HLA-I/KIR binding screening data, with KIR2DS1ζ$^+$ and KIR2DL1ζ$^+$ Jurkat cells being both activated by 721.221- cell lines expressing HLA-C2 ligands, and KIR2DL1ζ$^+$ Jurkat cells being more strongly activated than KIR2DS1ζ$^+$ Jurkat cells.

KIR2DS1 and KIR2DL1 binding to HLA-C2 molecules is peptide-dependent. Several studies have revealed that peptides presented by HLA class I molecules can influence KIR binding[19–21,25]. We therefore investigated whether specific peptides presented by the HLA-C2 allele HLA-C*06:02 can impact KIR2DS1- as well as KIR2DL1-binding. To prevent 721.221-HLA-C*06:02 cells from presenting self-peptides onto HLA-C*06:02, 721.221-HLA-C*06:02 cells with a knock-out for TAP1 were produced. This allowed for the controlled identification of externally added peptides that bind to and stabilize HLA-C*06:02 expression on the cell surface. HLA stabilization was quantified by measuring HLA expression on 721.221.TAP1KO-HLA-C*06:02 cells using flow cytometry.

We first tested 19 synthetic peptides that had been previously described to bind to HLA-C*06:02[26]. Furthermore, 568 overlapping peptides spanning the entire HIV-1 clade B sequence peptides (346 18aa-long peptides covering the entire HIV-1 consensus sequence and 222 decametric peptides overlapping by 9 amino acid and covering p24 GAG) were assessed for their potential to stabilize HLA-C*06:02. Peptides inducing a robust increase of HLA-C*06:02-expression (MdFI higher than 2 S.D. above the mean of non-stabilizing control peptide (LLRHHNLIY)) were defined as HLA-C*06:02-binding peptides, resulting in the identification of 20 peptides presented by HLA-C*06:02 (Fig. 3a). The peptides included 14 of the 19 previously described synthetic HLA-C*06:02-binding peptides and 6 novel HIV-1-derived peptides. Most of the peptides stabilizing HLA-C*06:02 showed the same binding motif consisting of a phenylalanine in position 1 of the peptide sequence (15/20), an arginine in position 2 of the peptide sequence (15/20) and an aliphatic amino acid (valine, leucine or isoleucine) in position 9 of the peptide sequence (16/20), which is in agreement with the previously defined binding motif for HLA-C*06:02[26–28] (see Supplementary Table 1).

The 20 selected peptides that stabilized HLA-C*06:02 expression were subsequently tested for their ability to engage KIR2DS1- and KIR2DL1 by co-incubating KIR2DS1ζ$^+$ and KIR2DL1ζ$^+$ Jurkat reporter cells with 721.221-TAP1KO- HLA-C*06:02 cells loaded with the respective peptides (Fig. 3b). Only one out of the 20 tested peptides, SRGPVHHLL (HLA-Cw6-SV9), showed a significant increase of reporter cell activity for both KIR2DS1ζ$^+$ and KIR2DL1ζ$^+$ Jurkat cell lines. None of the 6 identified HLA-C*06:02-binding HIV-1 peptides induced any activation of Jurkat reporter cells lines. The SRGPVHHLL peptide furthermore increased the reporter cell activity of both KIR2DS1ζ$^+$ and KIR2DL1ζ$^+$ Jurkat cells in response to TAP-competent 721.221. HLA-C*06:02 cells (Fig. 3c). Blocking experiments (Fig. 3d) were performed by pre-incubation of 721.221. TAP1KO-HLA-C*06:02 cells pulsed with the peptide SRGPVHHLL with an HLA-C blocking antibody (clone 6A4, IgM) before incubation with KIR2DS1ζ$^+$ and KIR2DL1ζ$^+$ Jurkat cells and resulted in the abrogation of reporter cell activity, confirming that KIR2DS1ζ$^+$ and KIR2DL1ζ$^+$ Jurkat cells activation was HLA/peptide-dependent.

To further confirm our findings, HLA-C*06:02 tetramers folded with the peptide SRGPVHHLL and conjugated to the fluorescent molecule PE (referred as HLA-C*06:02-SRGPVHHLL-PE) were used to stain the KIR2DS1ζ$^+$ and KIR2DL1ζ$^+$ Jurkat cells (Fig. 3e). A strong increase of PE MdFI signal was observed when the KIR2DS1ζ$^+$ and KIR2DL1ζ$^+$ Jurkat cells were stained with the HLA-C*06:02-SRGPVHHLL-PE tetramer. Taken together, out of the 587 peptides tested, we identified one synthetic peptide, SRGPVHHLL, which stabilized HLA-C*06:02-expression and induced strong and functionally relevant binding of KIR2DS1 and KIR2DL1.

Amino acid variations within the HLA-C*06:02-restricted SRGPVHHLL peptide impact KIR2DS1 binding. To determine the peptide residue important for modulating KIR2DS1- and KIR2DL1-binding, various amino acid substitutions were introduced in the SRGPVHHLL peptide sequence. A panel of 8 peptides (see Supplementary Table 2) was synthetized with amino acid substitutions at P2 or P9, identified previously as anchor

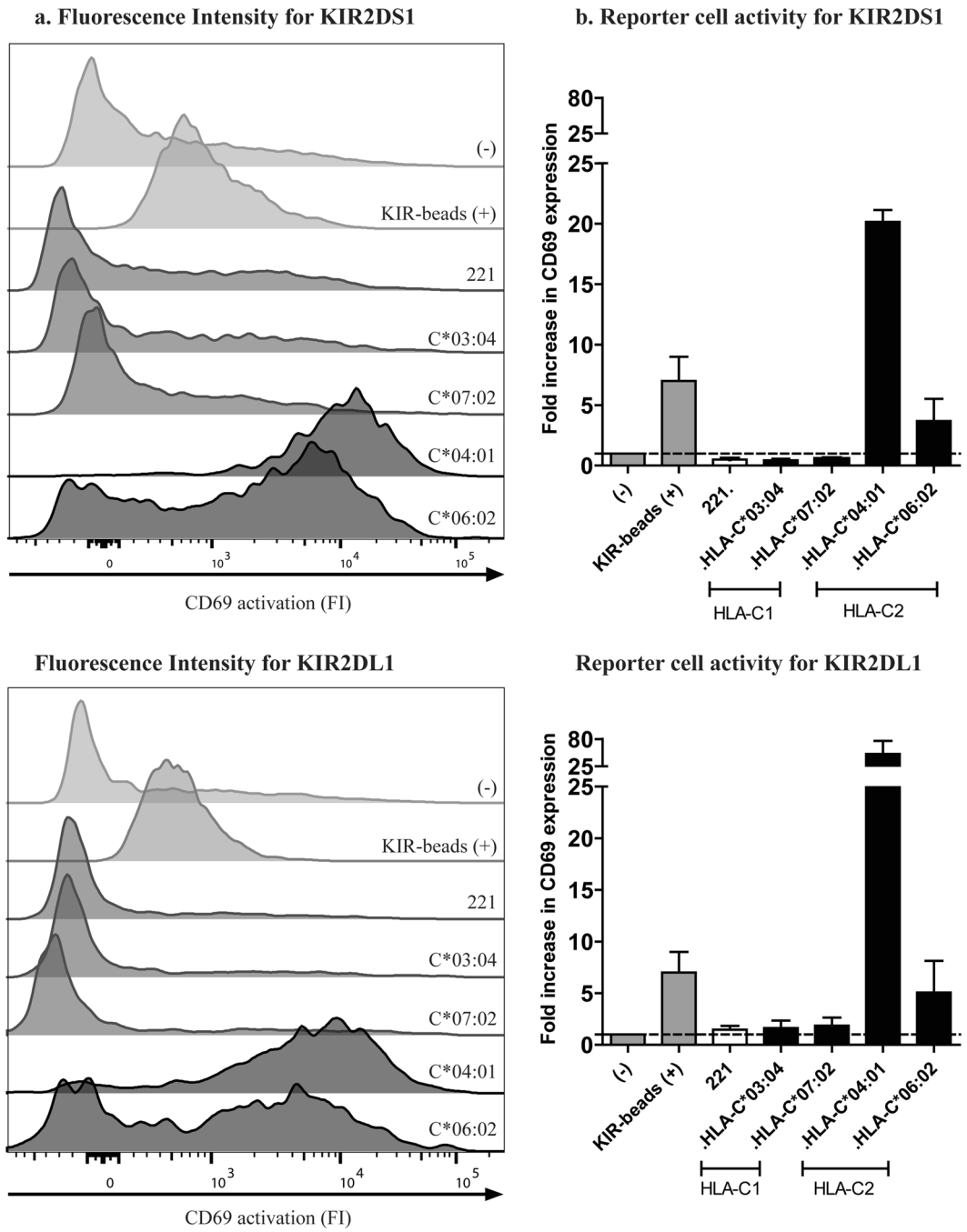

Figure 2. Binding of KIR2DS1 and KIR2DL1 reporter cell lines to HLA-C cell lines. KIR2DS1ζ$^+$ or KIR2DL1ζ$^+$ Jurkat cells were incubated with 721.221 cell lines transduced with HLA-C*03:02 or HLA-C*07:02 (HLA-C1), HLA-C*04:01 or HLA-C*06:02 (HLA-C2). KIR2DS1ζ$^+$ or KIR2DL1ζ$^+$ Jurkat cells alone were used as negative control (−) and KIR2DS1ζ$^+$ or KIR2DL1ζ$^+$ Jurkat cells pulsed with KIR2D-coupled beads were used as positive control (+). (**a**) Representative flow cytometry histogram showing Fluorescence Intensity (FI) for CD69 activation for KIR2DS1ζ$^+$ and KIR2DL1ζ$^+$ Jurkat cells. (**b**) Bar graph showing fold increase in CD69 expression (MdFI of the sample divided by the MdFI of KIR2DS1ζ$^+$ or KIR2DL1ζ$^+$ Jurkat cells stained in the absence of target cells). Each bar represents median +/− interquartile range of 3 independent experiments.

residues for HLA-C*06:02-binding[26–28], or P7, a position described as important for KIR recognition[19–21]. The amino acid substitutions were selected to cover the principal categories of amino acids according to their side chain (polar, apolar, neutral, acidic, basic). Peptides were first tested for HLA-C*06:02 stabilization and 7 out of the 8 peptides were identified to stabilize HLA-C*06:02-expression, demonstrating that most single amino acid substitution did not affect HLA-C*06:02-binding (Fig. 4a). The peptide R_2A/L_9A did not show any stabilization of HLA-C*06:02, suggesting that amino acid substitutions in both anchor position (P2 and P9) abrogated HLA-C*06:02-binding. Subsequently, the peptide variants were tested for their ability to modify KIR2DS1ζ$^+$

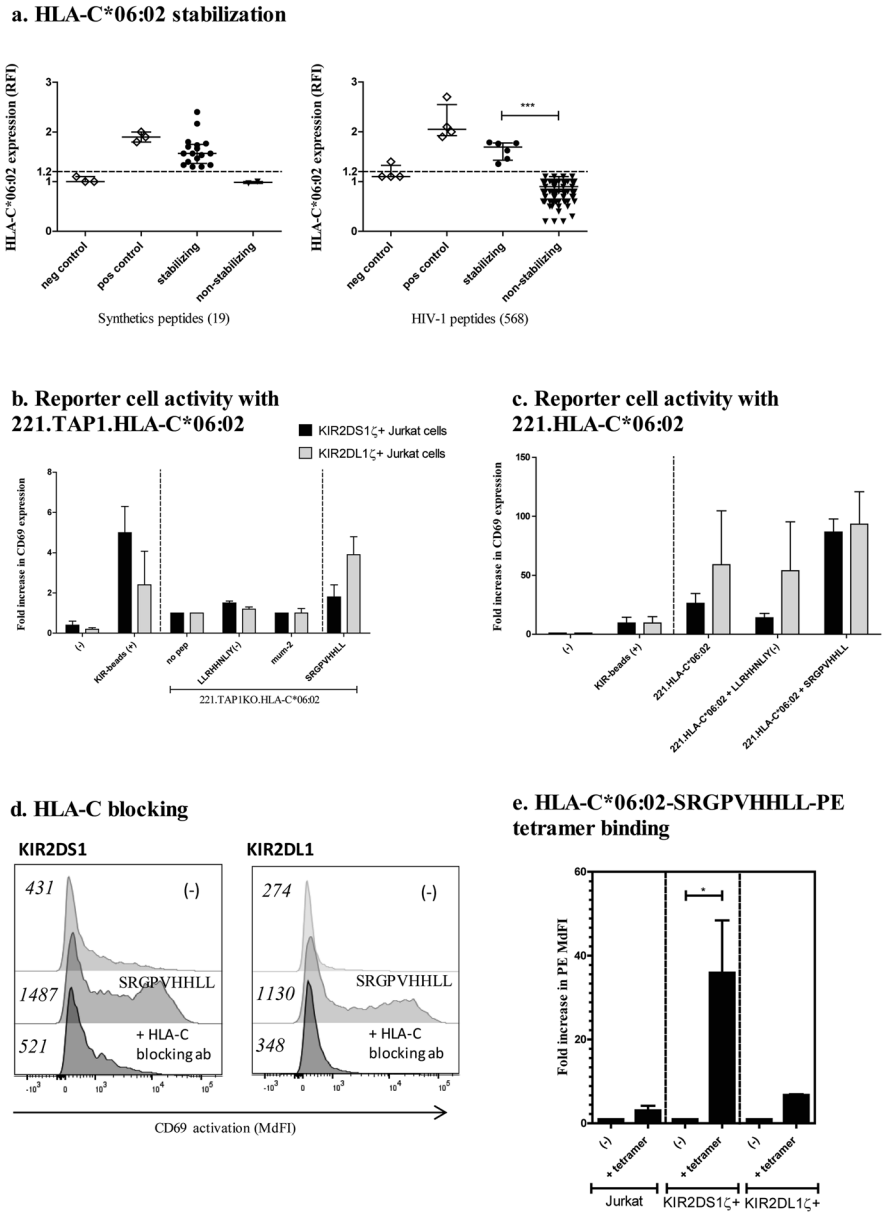

Figure 3. Impact of peptide presented by HLA-C*06:02 on KIR2DS1 and KIR2DL1 binding. (**a**) Quantification of HLA-C*06:02 stabilization of 721.221-TAP1KO-C*06:02 pulsed with 19 different synthetic peptides and 568 HIV-1 clade B peptides. HLA-C*06:02 surface levels were determined by flow cytometry using an anti-pan-HLA class I antibody (clone W6/32). Peptides were added at a saturating concentration of 200 μM. Relative fluorescence intensity (RFI) was calculated as the pan-HLA MdFI of the sample divided by the pan-HLA MdFI of 721.221-TAP1KO-C*06:02 cells stained in the absence of peptide. Binding peptides were determined as $(\text{Mean} + \text{S.D.})_{\text{MFI sample}} > (\text{Mean} + 2*\text{S.D.})_{\text{MFI LIY}}$. The dotted line represents the cut-off set to determine the peptides binding to HLA-C*06:02. The screening was performed one time and the selected peptides were tested in three independent experiments. (**b**) Bar graphs showing fold increase in CD69 expression for KIR2DS1ζ$^+$ (black bar) and KIR2DL1ζ$^+$ (grey bar) Jurkat cells when co-incubated with 721.221-TAP1KO-C*06:02 pulsed with different peptides (MdFI of the sample divided by the MdFI of KIR2DS1ζ+ or KIR2DL1ζ+ Jurkat cells co-incubated with 721.221-TAP1KO-C*06:02 in the absence of peptide). (**c**) Bar graphs showing fold increase in CD69 expression for KIR2DS1ζ$^+$ (black bar) and KIR2DL1ζ$^+$ (grey bar) Jurkat cells when co-incubated with 721.221-C*06:02 pulsed with different peptides (MdFI of the sample divided by the MdFI of KIR2DS1ζ$^+$ or KIR2DL1ζ$^+$ Jurkat cells alone). (**d**) HLA-C blocking antibody (6A4) abrogated CD69 activation of the KIR2DS1ζ$^+$ and KIR2DL1ζ$^+$ Jurkat cells after pre-incubation with the indicated target cells and subsequent incubation with the KIR2DS1ζ$^+$ or KIR2DL1ζ$^+$ Jurkat cells. The experiments were repeated three times independently and a representative example is shown with the mean of the CD69 FI. (**e**) HLA-C*06:02-SRGPVHHLL -PE tetramer staining of Jurkat- β2mKO, KIR2DS1ζ$^+$ or KIR2DL1ζ$^+$ Jurkat cells. Results are shown as fold increase in PE MdFI (MdFI of the reporter cell line stained with the tetramer divided by the MdFI of the reporter cell line alone). For each bar graphs, the results are shown as median of three independent experiments +/− interquartile range.

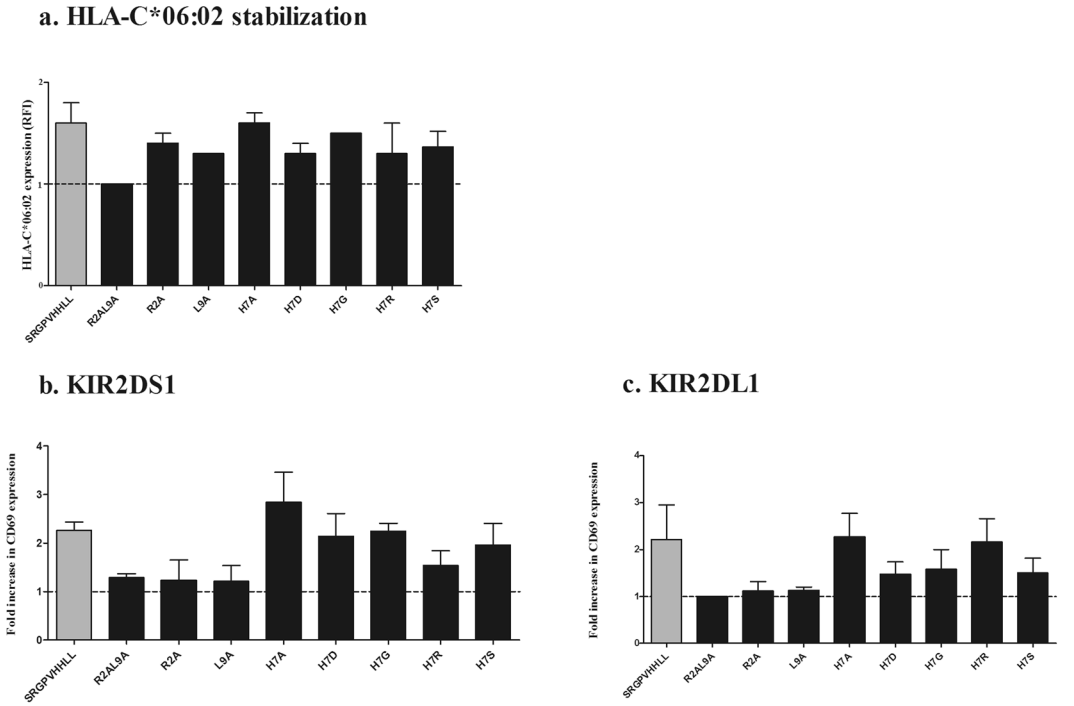

Figure 4. Impact of amino acid modification of the peptide SRGPVHHLL presented by HLA-C*06:02 on KIR2DS1 and KIR2DL1 binding. (**a**) Quantification of HLA-C*06:02 stabilization of 721.221.TAP1KO- HLA-C*06:02 pulsed with the indicated peptides. Peptides were added at a saturating concentration of 200 μM. Of note, the median +/−S.D. of R2AL9A, L9A and H7G were respectively 1 ± 0, 1.3 ± 0.057 and 1.5 ± 0.057 (**b**) and (**c**) Bar graph showing fold increase in CD69 for KIR2DS1ζ$^+$ or KIR2DL1ζ$^+$ Jurkat cells when co-incubated with 721.221-TAP1KO-C*06:02 pulsed with different peptides (MdFI of the sample divided by the MdFI of KIR2DS1ζ$^+$ or KIR2DL1ζ$^+$ Jurkat cells co-incubated with 721.221-TAP1KO-C*06:02 in the absence of peptide). Each bar represents median +/− interquartile range of 3 independent experiments.

and KIR2DL1ζ$^+$ binding when presented by HLA-C*06:02 (Fig. 4b and c). The peptide R_2A/L_9A, which did not stabilize HLA-C*06:02, was used as an additional negative control. The peptides R_2A and L_9A, which stabilized HLA-C*06:02-expression, decreased both KIR2DS1ζ$^+$ and KIR2DL1ζ$^+$ Jurkat cell activity to the same level as the negative control peptide (LLRHHNLIY), showing that single substitution in P2 or P9 abrogated KIR2DS1 as well as KIR2DL1 binding. The peptides with the sequence changes H_7A, H_7G and H_7S did not affect KIR2DS1ζ$^+$ and KIR2DL1ζ$^+$ Jurkat cell reporter activity compared to wild type peptide. Finally, the peptide with the H_7R substitution slightly decreased the reporter cell activity of KIR2DS1ζ$^+$ Jurkat cells, but not the activity of KIR2DL1ζ$^+$ Jurkat cells, while the peptides with the H_7D and H_7S substitutions slightly decreased the reporter cell activity of KIR2DL1ζ$^+$ Jurkat cells, but not of KIR2DS1ζ$^+$ Jurkat cells. In summary, we demonstrated that binding of KIR2DS1 and KIR2DL1 to the SRGPVHHLL peptide presented by HLA-C*06:02 was modulated by the peptide sequence.

SRGPVHHLL presented by HLA-C*06:02 triggers degranulation of primary KIR2DS1+NK cell clones. The functional consequences of peptide-dependent engagement of activating KIRs remains insufficiently understood. The effects of the SRGPVHHLL peptide presented by HLA-C*06:02 on KIR2DS1 binding was therefore investigated by performing NK cell degranulation assays using primary KIR2DS1(+) KIR2DL1(−) and KIR2DS1(−) KIR2DL1(−) NK cell clones derived from KIR2DS1+ HLA-C1/C1 individuals (see Supplementary Fig. 1 for gating strategy and characterization of NK cells clones). As displayed in Fig. 5a and b, 721.221-TAP1KO- HLA-C*06:02 cells pulsed with the SRGPVHHLL peptide induced strong degranulation of KIR2DS1(+) KIR2DL1(−) NK cell clones, as measured by the percentage of CD107a(+) NK cells, compared to 721.221.TAP1KO-HLA-C*06:02 cell alone or pulsed with the control peptide LLRHHNLIY. In contrast, KIR2DS1/KIR2DL1 double-negative NK cell clones were not activated by the peptide SRGPVHHLL. Moreover, HLA-C*06:02-tetramers refolded with the SRGPVHHLL peptide stained KIR2DS1(+) KIR2DL1(−) NK cell clones, while no binding to KIR2DS1(−) KIR2DL1(−) NK cell clones was observed (Fig. 5c). Of note, as previous studies demonstrated that the co-expression of the inhibitor receptors KIR2DL2/KIR2DL3, KIR3DL1 and NKG2A can affect the outcome of KIR2DS1(+) NK cell degranulation[29], we phenotyped all NK cell clones for these receptors. The presence or absence of KIR2DL2/KIR2DL3, KIR3DL1 and NKG2A on these clones did not affect the results (data not shown). All together, these data demonstrate that the HLA-C*06:02 peptide SRGPVHHLL enabled KIR2DS1-binding and resulted in the activation of KIR2DS1(+), but not KIR2DS1(−) NK cell clones derived from the same individual.

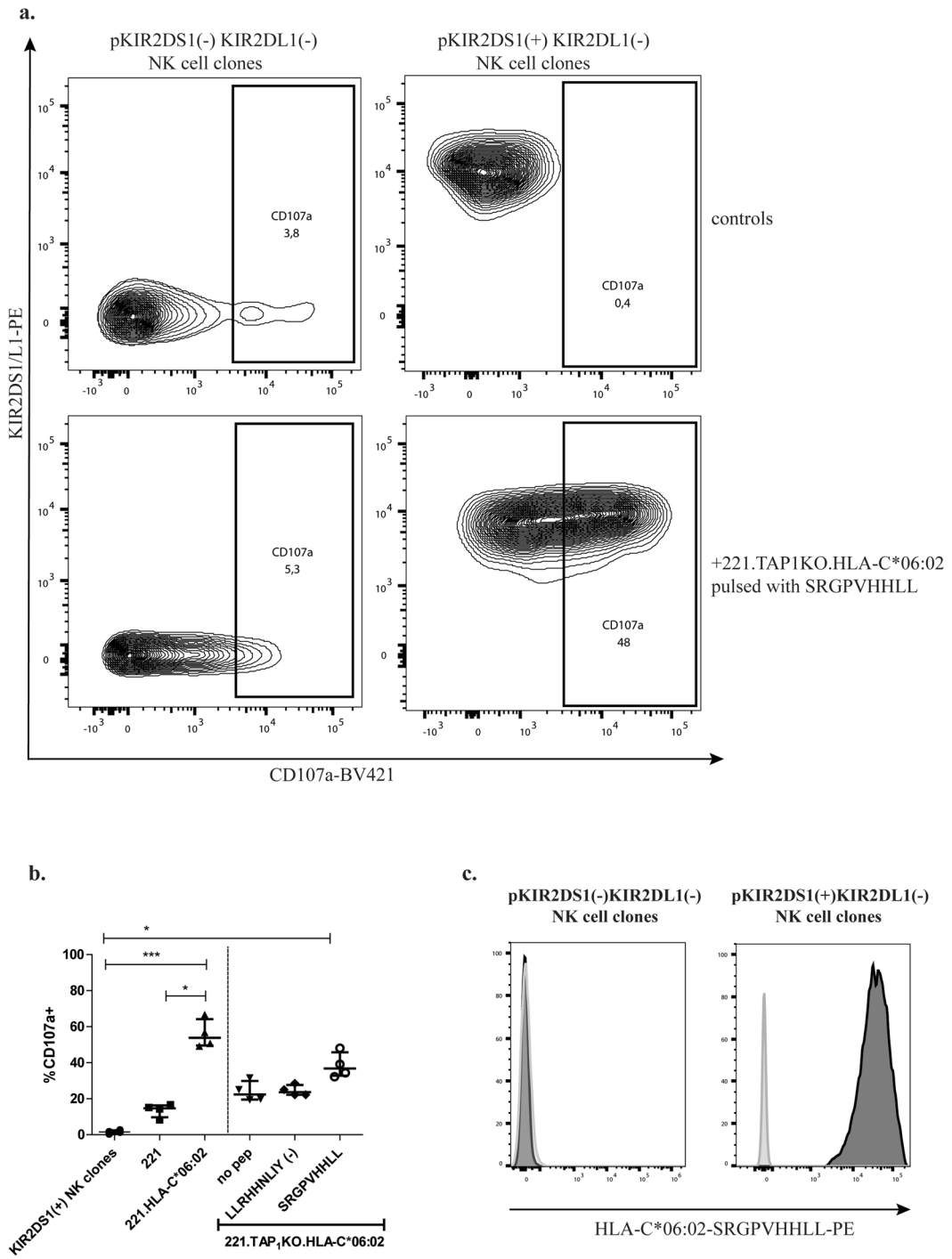

Figure 5. Functional impact of peptide SRGPVHHLL on primary KIR2DS1(+) KIR2DL1(−) and KIR2DS1(−)KIR2DL1(−) NK cell clones. (**a**) Representative example of CD107a production by primary KIR2DS1(+) KIR2DL1(−) and KIR2DS1(−) KIR2DL1(−) NK cell clones cultured alone or in presence of 721.221.TAP1KO- HLA-C*06:02 pulsed with 100 µM of the peptide SRGPVHHLL (**b**) Paired comparison of the percentage of CD107a NK cells after 5hrs stimulation of clonal KIR2DS1(+) KIR2DL1(−) NK cells with target cells. The experiments were repeated 4 times independently and showed are the median +/− interquartile range. (**c**) Representative example of HLA-C*06:02- SRGPVHHLL -PE tetramer staining of primary KIR2DS1(+) KIR2DL1(−) or KIR2DS1(−) KIR2DL1(−) NK cell clones. Primary KIR2DS1(+) KIR2DL1(−) NK cell clones incubated without the tetramer are represented in grey whereas the cells stained with the tetramer are represented in black.

Discussion

The factors that determine the engagement of the activating NK cell receptor KIR2DS1 remain incompletely understood despite a growing number of genetic studies showing associations between KIR2DS1 and the outcome of various human diseases. In this study, we investigated the HLA class I molecules and HLA class I-presented peptides enabling KIR2DS1-binding and their influence on KIR2DS1(+) NK cell function. We demonstrate that KIR2DS1-binding is narrowly restricted to HLA-C2 molecules whereas KIR2DL1 showed a broader binding specificity for HLA-C2 but also for some HLA-C1 and HLA-B molecules. HLA-C2-presented peptides modulated both KIR2DS1- and KIR2DL1-binding to HLA-C2, and we identified one HLA-C*06:02-presented peptide (SRGPVHHLL) that strongly engaged KIR2DS1 and also KIR2DL1 binding. This synthetic peptide, predicted to bind HLA-C*06:02, did not correspond to any known human or viral sequence, and strongly activated primary KIR2DS1(+) NK cell clones in a peptide sequence-specific manner. Taken together, these data demonstrate peptide-dependent activation of the activating NK cell receptor KIR2DS1 and its inhibitory counterpart KIR2DL1.

A broad HLA class I-binding screen performed in this study demonstrated that KIR2DL1 and KIR2DS1 exhibited very similar binding specificities to HLA-C2 molecules, consistent with previous studies[16,17,22]. However KIR2DL1 showed a higher binding affinity for these HLA-C2 ligands, and a broader HLA class I binding specificity than KIR2DS1, as it also bound weakly to eight HLA-C1 complexes (HLA-C*01:02; *03:02; *03:03; *03:04; *07:02; *08:01; *12:03; *16:01) as well as two HLA-B complexes (HLA-B*46:01 and HLA-B*73:01). Given the opposite functions of the activating KIR2DS1 receptor and the inhibitory KIR2DL1 receptor, a very restricted set of ligands for the activating NK cell receptor KIR2DS1 might limit the risks of auto-immune reaction. Furthermore, both KIR2DS1 and KIR2DL1 shared the same pattern of peptide-specific recognition, which is consistent with previous studies[17,22,23,48] as they bound to the same HLA-C*06:02-presented peptide SRGPVHHLL. The functional assays performed using KIR2DS1ζ^+ and KIR2DL1ζ^+ Jurkat reporter cells also showed that KIR2DL1 had a stronger binding affinity than KIR2DS1, consistent with previous studies showing that inhibitory receptors have a stronger binding affinity than their corresponding activating NK cell receptor[17,30] for the same ligand. Of note, KIR2DL1 and KIR2DS1 are not always present at the surface of the same NK cell as KIR receptors are expressed stochastically, and it has been shown that around 10% of circulating NK cells expressed KIR2DS1 in absence of KIR2DL1[31]. Our data suggest that this subset of KIR2DS1(+) KIR2DL1(−) NK cells is able to mediate an effective effector function in response to their ligands. Notably, the tetramer staining performed using HLA-C*06:02 molecules refolded with the peptide SRGPVHHLL showed higher binding affinity for KIR2DS1 compared to KIR2DL1. This result might be linked to the observation that KIR2DS1 can assemble in larger clusters than KIR2DL1 at the surface of cells[32], potentially favoring a stronger binding of HLA class I tetramers. Overall, our data showed that the NK cell receptors KIR2DS1 and KIR2DL1 shared the same ligand specificity for HLA-C2-presented peptides, but differed in their binding affinity.

Several studies using crystal structures of KIR2DL1[21], KIR2DL2[19], KIR2DL3[20] and KIR2DS2[25] have described that the KIR binding interactions with HLA class I presented epitopes are centered to the COOH-terminal end of the peptide which corresponds to peptide residues P7-P8[33]. In contrast, the specific recognition of HLA-presented peptide by TCR depends on the sequence of the entire peptide and is centered at the P4-P6 positions[34]. Our results showed that peptide residue P7 of the HLA-C*06:02 presented peptide SRGPVHHLL can modulate KIR2DS1 and KIR2DL1 binding, as previously described[22], but that amino acid changes in residues P2 and P9 can also impact the binding of KIR2DS1 and KIR2DL1. The residues P2 and P9 are defined as anchor residues for HLA-C*06:02 binding[26–28]. Modifications of these residues may induce conformational changes of the peptide SRGPVHHLL presented by HLA-C*06:02, which can abrogate the binding to KIR2DS1 and KIR2DL1. The resolution of the crystal structure of KIR2DS1 in conjunction with HLA-C molecules presenting specific peptides will help clarifying these interactions. In summary, our results suggest that the HLA-C*06:02-presented peptide SRGPVHHLL can modulate KIR2DS1 and KIR2DL1 in a peptide sequence-specific manner.

Very few studies have investigated the ability of HLA class I-presented peptides to facilitate the binding of activating KIRs. Here we demonstrate that KIR2DS1-activation can be modulated by HLA-C2-presented peptides; however, despite screening over 500 virus-derived peptides, we did not identify any viral peptides binding to KIR2DS1. The peptide SRGPVHHLL identified to enable strong KIR2DS1-binding to HLA-C*06:02 was derived from a synthetic peptide library predicted to bind to HLA-C*06:02[26], and does not match any known viral or human epitope. Several HIV-1-derived peptides presented by their respective HLA class I molecules have been demonstrated to modulate the binding of inhibitory KIRs, including KIR2DL2[35,36], KIR2DL3[37] and KIR3DL1[38,39]. Thus, it is remarkable that none of these peptides was able to engage the binding of KIR2DS1. Studies have indicated that viruses are able to select for sequence variants that enhanced the binding of inhibitory KIRs and thus inhibit the effector function of KIR+ NK cells, suggesting that viruses are able to escape NK cell-mediated immune pressure[38–40]. It might therefore be possible that HIV-1 has eliminated sequences that do allow binding to the activating KIR2DS1 receptor when presented by the HLA-C*06:02 molecule. To our knowledge, the only viral peptide presented by HLA class I and binding to an activating KIR that has been identified is a vaccinia virus-derived peptide in complex with HLA-A*11 binding to KIR2DS2[25]. Additionally, direct binding of primary activating KIR2DS1+ NK cells to virally infected cells has only been shown for EBV-transformed 221.HLA-C*04:01[17], but no specific viral peptide was identified. Taken together, viruses might thus have evolved specific mechanisms to avoid recognition of viral peptides by activating NK cell receptors.

The role of activating KIRs has been extensively studied in the context of allogeneic stem cell transplantation to treat leukemia, especially in the setting of KIR ligands-mismatched donor/recipient pairs[41–44]. In particular, donor-derived KIR2DS1+ NK cells were shown to efficiently kill HLA-C2+ leukemia blasts, which indicate that activating KIRs can have a role in mediating anti-leukemic or anti-cancer effects[29]. The HLA-C*06:02 presented peptide SRGPVHHLL identified in this study mediated strong activation of primary NK cell clones, suggesting that this peptide could be potentially used to label specific tumors in order to enhance anti-tumor cytotoxic

activity by KIR2DS1(+) NK cells. The use of the synthetic SRGPVHHLL peptide as an enhancer for KIR2DS1(+) NK cell-mediated cytotoxicity might therefore provide a new perspective for NK cell immunotherapy.

Methods

Cell lines and PBMC. The HLA-class-I deficient 721.221 B cell line was used to produce the cell lines 721.221-HLA-C*06:02, -HLA-C*04:01, -HLA-C*03:04 and -HLA-C*07:02 using methods already described[46]. The 721.221.TAP1KO-HLA-C*06:02 cell line was generated by knocking out the TAP1 gene using CRISPR/CAS9 technology. Briefly, 721.221-HLA-C*06:02 were transduced with lentiCas9-Blast (Addgene plasmid # 52962) and selected in 5 μg/mL blasticidin S (Sigma-Aldrich). 721.221-HLA-C*06:02.Cas9 cells were transduced with TAP1 gRNA lentivirus (Addgene plasmid # 52962)), selected in 200 μg/ml Neomycin (Sigma-Aldrich) and sorted for loss of HLA expression. LentiCas9-Blast and TAP1 gRNA lentiviruses were kindly provided by Feng Zhang[45]. The Jurkat cell lines (clone E6, ATCC) were used to generate Jurkat-β2 mKO-KIR2DS1 cells (referred as KIR2DS1ζ$^+$ Jurkat cells) and Jurkat-β2mKO-KIR2DL1 cells (referred as KIR2DL1ζ$^+$ Jurkat cells). The Jurkat cell lines were produced via knocking out of the β2m as already described[46]. KIRζ chimeric constructs with the extracellular domain of KIR2DS1*002 or KIR2DL1*001 (Gene Art) were cloned into a lentiviral transfer vector containing an SFFV promoter and IRES-driven puromycin resistance. Lentiviral supernatant produced by three-plasmid transfection (psPAX2, VSV-G and transfer vector) of 293T cells via lipofectamine[37] was obtained to transduce Jurkat-β2mKO cells. 72 h post-transduction, the cell lines Jurkat- β2mKO.KIR2DS1 and Jurkat- β2mKO.KIR2DL1 were selected in 2 μg/ml Puromycin (Sigma-Aldrich) and sorted for high expression of KIR. Human primary blood mononuclear cells (PBMC) were isolated from healthy donors recruited at the University medical Centre Hamburg-Eppendorf, using density centrifugation. Each participant gave informed consent prior to enrollment. All cell lines were cultivated at 37 °C under 5% CO2 in RPMI medium 1640 (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich), 2,500 U/ml penicillin, 2,500 μg/ml streptomycin, and 100 mML-glutamine (Cellgro) (referred as RP10). All methods were performed in accordance with the relevant guidelines and regulations and approved by the ethical commission of the Ärztekammer Hamburg.

Antibodies. The following antibodies were used for cellular assays: anti-HLA-ABC-APC (clone W6/32, eBioscience), anti-CD3-BU737 (clone UCHT1, BD Bioscience), anti-CD3-PerCyP.5.5 (clone UCHT1, BioLegend), anti-CD19-BV510 (clone HIB19, BioLegend), anti-CD56-BU395 (clone NCAM16.2, BD Bioscience), anti-CD16-BV785 (clone 3G8, BioLegend), anti-KIR2DL1/S1/L3/S3/L5/S5-PE (clone HP-MA4, eBioscience), anti-KIR2DL1/S1-PE (clone 11PB6, Miltenyi), anti-KIR2DL1-FITC (clone REA284, Miltenyi), anti-KIR2DL3-APC (clone DX27, Miltenyi), anti-KIR3DL1/S1-BV421 (clone DX9, BioLegend), anti-NKG2A-PE-Cy7 (clone Z199, Beckman), anti-NKG2D-APC (clone 1D11, BioLegend), goat anti-human IgG(Fc) F(ab´)-PE (Life Technologies), anti-CD69-BV421 (clone FN50, BioLegend), anti-CD107a-BV421 (clone M4A3, BioLegend). For blocking assays we pre-incubated the samples with anti-HLA-C antibodies (clone 6A4, IgM, kindly provided by Prof. L. Moretta, Istituto Giannina, Genova, Italia) diluted 1/20 for 20 min at 37 °C.

KIR-Fc binding assay to HLA-I coated beads. Screening of classical HLA-I coated beads was performed using LABScreen Single Antigen HLA Class I - Combi (One Lambda) according to the manufacturer's instructions. 40 μl of indicated concentration of KIR2DS1-Fcs (allele KIR2DS1*002) and KIR2DL1-Fcs (allele KIR2DL1*001) (R&D system) diluted in PBS were added to a 96-well plate and incubated with a mixture of 97 classical anti-human HLA-A, B, C beads for 30 mn at room temperature. The plate was washed and the samples were incubated with goat- anti-human IgG-PE secondary antibody for 30 mn at room temperature. Finally, KIR-Fc binding to the beads was analyzed on a Bio-Plex 200 (Bio-Rad Laboratories). Results for KIR-Fc binding to HLA-I coated beads were presented as Median Fluorescence Intensity (MdFI) of the KIR-Fc binding.

Peptide binding assay for HLA-C stabilization. HLA Class I -stabilization assays were performed as previously described[46]. 721.221.TAP1KO- HLA-C*06:02 were incubated 24 hrs in serum-free RPMI 1640 to remove any remaining peptides from the RP10 medium. Cells were washed twice and pulsed with 200 μM of the indicated peptides for 20 hrs at 37 °C in serum-free medium (referred as "R0"). The staining was performed using the Zombie Aqua fixable viability kit (BioLegend) following the manufacturer´s instructions and anti HLA-ABC-APC to quantify HLA class I expression. After fixation in 4% paraformaldehyde, samples were analyzed by flow cytometry (BD LSR Fortessa). The 721.221-TAP1KO- HLA-C*06:02 incubated with no peptide as well as the HCV peptide, LLRHHNLIY were used as negative control. As positive controls, 721.221-HLA-C*06:02 cell line, which stably expresses HLA-C*06:02, and the MUM-2 derived peptide, FRSGLDSYV, already described as HLA-C*06:02 binder[26] were used.

KIR-Jurkat reporter cell assay. KIRζ chimeric constructs for the generation of the KIR2DL1ζ$^+$ Jurkat reporter cells consisted of the extracellular and transmembrane domains of KIR2DL1*001 linked to the cytoplasmic tail of CD3ζ. KIR2DS1ζ$^+$ Jurkat reporter cells contained the extracellular domain of KIR2DS1*002, the transmembrane domains of KIR2DL1*001 linked to the cytoplasmic tail of CD3ζ. A sequence coding for the Zs Green protein was added to the KIR2DS1 chimeric construct to allow the differentiation between the KIR2DL1 and KIR2DS1 reporter cell line by GFP signal. Ligand engagement of KIR2DL1 or KIR2DS1 resulted in an activating signal that triggered CD69 expression. Measurement of KIR binding was assessed as Median Fluorescence Intensity (MdFI) of CD69. Reporter cells line were cultured overnight in RP20 (RPMI supplemented with 20% FBS) at 2.5*10^5 cells/ml to reduce background activation. KIR2DS1ζ$^+$ Jurkats cells or KIR2DL1ζ$^+$ Jurkats cells were co-incubated with target cells pulsed with peptides at a ratio effector/target 1/10 for 3 hrs at 37 °C under 5% CO2 in RP20. Cells were washed and stained for anti-CD3-BUV737, live/dead-BV510, anti-KIR2DL1/S1/L3/S3/L5/S5-PE (HPMA4) and anti-CD69-BV421 20 mn at room temperature in the dark. After fixation in 4%

paraformaldehyde, the samples were analyzed by flow cytometry (BD LSR Fortessa). KIR2DS1ζ$^+$ or KIR2DL1ζ$^+$ -Jurkat cells were used as negative control and KIR2DS1ζ$^+$ or KIR2DL1ζ$^+$ -Jurkat cells pulsed with 10 µl of HPMA4-conjugated beads (Dynabeads M-450 Tosylactivated, Invitrogen, conjugated following the manufacturer's instructions) were used as positive control.

Generation of clonal NK cells. Primary NK cell clones were generated from PBMCs from healthy donors with a KIR2DS1+ and HLA-C1 homozygous genotype using methods described[46]. We isolated NK cells using EasySep™ Human NK Cell Enrichment Kit (StemCell) following manufacturers protocol. NK cells were cultivated overnight in RP10 supplemented with 500 U/ml IL2. Next day, they were resuspended in cloning medium consisting of RPMI supplemented with 10% fetal bovine serum (Sigma-Aldrich), 5% human serum (Sigma-Aldrich), 2 mM L-glutamine (Gibco), 1X MEM-NEAA (Gibco), 1X sodium pyruvate (Gibco), 100 µg/mL Primocin™ (Invivogen), 500 U/mL IL-2 (Sigma Aldrich), with the addition of the following four cytokines: 5 ng/mL IL-15 (Peprotech), 10 ng/mL IL-12 (Peprotech), 40 ng/mL IL-18 (Peprotech), and 20 ng/mL IL-21 (Peprotech). NK cells were stained for anti-CD3-BUV737, anti-CD19-BV510, anti-CD56-BUV395, anti-CD16-BV785, anti-KIR2DL1-S1-PE (EB6) and anti-KIR2DL1-FITC (REA284) and sorted for single cells in 96 well plates using FACS ARIA Fusion to produce 2 different subsets of NK cell clones: KIR2DS1(+) KIR2DL1(−) and KIR2DS1(−) KIR2DL1(−). After sorting, 100 µl of feeders cells consisting of irradiated K562 cells expressing mbIL-15 and CD137L (kind gift from Dario Campana[47]) and irradiated allogeneic PBMC homozygous for HLA-C1 (ratio K562/PBMC 1/10) were added to the wells. After 10 days, 200 µL of NK cloning medium was added to the growth pellets. After 3 days, NK cell clones were harvested and transferred in 24 well plates with NK cloning medium and 500 U/ml IL2. After 3 supplementary days, NK cell clones were phenotyped and used for assays. To ensure proper functionality, NK cell clones were also phenotyped for NKG2A, KIR2DL2/3 and KIR3DS1.

NK cell degranulation assay. The day before the experiment, KIR2DS1(+) KIR2DL1(−) and KIR2DS1(−) KIR2DL1(−) NK cell clones were cultivated in NK cloning medium without IL-2 overnight to avoid background activation levels. The NK cell clones were co-incubated with target cells lines, previously pulsed with 100 µM peptide for 20 hrs, in the presence of 2 µl of anti-CD107a and 5 µg/ml brefeldin A (BioLegend) at an effector to target ratio of 1/5 in a 96 well plate in RP10 supplemented with 1 ng/ml IL-15 (Peprotec). After 5 hrs of incubation at 37 °C, 5% CO2, samples were washed and stained with anti-CD19-BV510, anti-CD3-PerCy5.5, anti-CD56-BUV395, anti-CD16-BV758, anti-KIR2DL1-FITC, anti-KIR2DS1/L1-PE (clone 11PB6) for 20 mn at room temperature. Cells were washed, fixed and flow cytometry analysis was performed on BD LSR Fortessa.

Tetramer staining. HLA-C*06:02- SRGPVHHLL-PE tetramer was provided by the NIH Tetramer Core Facility and used for staining of target cell lines. Briefly, $2*10^5$ cells of each target cell lines were incubated on ice for 5 mn in 50 µL blocking buffer (sterile PBS+ 10% human serum+ 3% fetal bovine serum (FBS)) in a 96 well plate. Cells were washed and resuspended in 50 µL blocking buffer and the tetramer was added at a dilution of 1/100 for 60 mn on ice in the dark. The samples were washed with FACS buffer (PBS+ 3% FBS) and stained with the corresponding antibodies for 30 mn on ice on the dark. After two washing rounds with FACS buffer, samples were fixed in 4% paraformaldehyde and analyzed by flow cytometry (BD LSR Fortessa). Measurement of HLA-C*06:02- SRGPVHHLL-PE tetramer binding was assessed as MdFI of PE.

Data acquisition, analysis and statistics. Flow cytometry data were analyzed using FlowJo software version 10.0.6 (Tree Star), and statistical analysis was performed using GraphPad Prism 5 (GraphPad Software). Each experiment was repeated independently 3 times except where stated otherwise. When indicated, statistical tests were performed assuming a non-parametric population using Kruskal-Wallis Test followed by post Dunn test analyzing all pairs of column. *,** and *** corresponded to $p < 0.05$; $p < 0.01$ and $p < 0.001$, respectively.

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Author Contributions

A.C., W.G.B., A.H., G.M. and M.A. conceived and designed the experiments. A.C., W.G.B. and A.H. performed the experiments. A.C. and G.M. analyzed the data. A.C., W.G.B., A.H., M.Z., S.L., G.M. and M.A. participated in discussion on the data and commented on the manuscript. A.C. and M.A. wrote the article.

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Chapter 2

Research Article

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Sequence variations in HCV core-derived epitopes alter binding of KIR2DL3 to HLA-C*03:04 and modulate NK cell function

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See Editorial, pages 237–239

Background & Aims: Both natural killer (NK) cells and human leukocyte antigen (HLA)/killer cell immunoglobulin like receptor (KIR) interactions have been shown to play an important role in the control, clearance and progression of hepatitis C virus (HCV) disease. Here we aimed at elucidating the effects of viral peptides derived from HCV on HLA stabilization, changes in KIR binding and primary NK cell function.

Methods: Transporter for antigen presentation-deficient 722.221 cells stably transfected with HLA-C*03:04 were used to screen 200 overlapping peptides, covering the non-structural protein 3 (NS3) and core protein of HCV genotype 1, for their ability to bind and stabilize HLA-C*03:04. Binding of KIR2DL3 to the HLA-peptide complex was assessed using a KIR2DL3-IgG fusion construct. Primary NK cells were isolated from healthy donors to investigate the effects of identified peptides on KIR2DL3$^+$ NK cell function.

Results: Thirty-one peptides able to stabilize HLA-C*03:04 were identified. One 9mer peptide, YIPLVGAPL, resulted in significantly higher KIR2DL3 binding to HLA-C*03:04$^+$ 722.221 cells and suppression of primary KIR2DL3$^+$ NK cell function. Interestingly this sequence exhibited a high frequency of mutations in different HCV genotypes. These genotype-specific peptides showed lower HLA-C*03:04 stabilization, decreased binding of the inhibitory KIR2DL3 and lower inhibition of NK cell function.

Conclusions: Taken together we show that a viral peptide derived from the core protein of HCV genotype 1 binding to HLA-C*03:04 results in a sequence-dependent engagement of the inhibitory NK cell receptor KIR2DL3, while the large majority of the remaining 30 HLA-C*03:04 binding HCV core peptides did not. These data show that sequence variations within HCV can modulate NK cell function, providing potential pathways for viral escape.

Lay summary: We identified a HCV peptide that dampens NK cell responses, and thereby possibly prevents killing of infected cells through this part of the innate immune system. This is facilitated via presentation of the viral peptide on HLA*03:04 to the inhibitory KIR receptor KIR2DL3 on NK cells. Naturally occurring sequence mutations in the peptide alter these interactions making the inhibition less efficient.

Introduction

Natural Killer (NK) cells play a pivotal role in the early defense against virus infections and malignancies [1,2], as they are part of the innate arm of the human immune system and able to respond rapidly against encountered pathogens without prior need of sensitization. The main NK cell function is the elimination of target cells by directed release of perforin and granzyme, or engagement of apoptosis-inducing receptors such as FAS [2]. Furthermore, NK cells can shape the following antigen-specific immune responses through the production of cytokines and chemokines or by interacting directly with other cells of the immune system, such as T cells, dendritic cells and monocytes [3,4].

Due to the potent nature of their effector functions, the activation of NK cells is tightly regulated via a plethora of activating and inhibiting receptors and their interaction with respective ligands [5,6]. One major group of receptors on NK cells are the killer cell immunoglobulin like receptors (KIRs), which interact mainly with human leukocyte antigen (HLA) class I molecules on the surfaces of other cells [7,8]. Both activating and inhibiting KIRs have been described. The binding of specific KIRs to their respective HLA ligand can be further modulated by the HLA class I-presented peptide [9]. These interactions have been shown to

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Abbreviations: HCV, hepatitis C virus; KIR, killer cell immunoglobulin like receptor; HLA, human leukocyte antigen; NK, natural killer; OLP, overlapping peptide.

play an important role in regulating the NK cell response against the human immunodeficiency virus (HIV), and also enable HIV-1 to escape NK cell recognition through the selection of viral sequence variations [10].

In the case of hepatitis virus infections, NK cells play an important role in the clearance of infection and disease progression [1]. A large number of studies have highlighted the importance of NK cells in viral hepatitis, focusing mainly on patients infected with hepatitis B virus (HBV) and hepatitis C virus (HCV) [11]. KIR-HLA interactions seem of particular importance in HCV infection as suggested by genetic association studies which linked HLA-C genotypes in conjunction with KIR2DL3 to viral clearance [12,13]. A more recent study further expanded on this, with the finding that KIR2DS3 is negatively associated with viral clearance [14]. The exact mechanism underlying these associations is not fully understood, but probably depends on the strength of the KIR/HLA interaction, which in case of KIR2DL3 and HLA-C alleles of the group 1 (HLA-C1) is rather weak, compared to other inhibition events [12]. HLA-C*03:04 is a common member of the HLA-C1 group, and is expressed at varying frequencies, ranging from 1% in Saudi Arabian populations all the way to 54% in Brazilians (allelfrequencies.net). In Caucasian populations in Germany, the range is between 14–21%. HLA-C*03:04 is a ligand for the above mentioned KIR2DL3, and the described role in HIV infection [16] might indicate further involvement in other viral infections.

While numerous HCV-derived $CD4^+$ and $CD8^+$ T cell epitopes have been described, no data are available on the role of HCV peptides presented by HLA class I molecules for KIR binding and KIR^+ NK cell function. Therefore, our aim was to investigate whether viral peptides derived from HCV core have an effect on the function of primary KIR^+ NK cells.

Materials and methods

Cell lines and human peripheral blood mononuclear cells

We used a previously described 721.221 human B-cell line, which has been stably transduced with ICP47, a herpes simplex virus (HSV) protein that blocks transporter for antigen presentation (TAP)-dependent loading of MHC class I molecules, and also transfected with HLA-C*03:04. Additionally we used the same cell line without ICP47 TAP-block. These cells were kept in RPMI medium 1640, supplemented with 10% heat-inactivated fetal calf serum, streptomycin and penicillin (R10). A *TAP*-knockout (KO) cell line based on the 721.221-C*03:04 cells was generated (221-C*03:04-*TAP1*-KO) using CRISPR/CAS technology [15] (plasmids were supplied by addgene). Cells were selected in R10 supplemented with puromycin, blasticidine and neomycin. Human primary blood mononuclear cells (PBMC) were isolated from healthy donors, using density centrifugation and either used directly or cryopreserved in liquid nitrogen until use. All donors gave informed consent.

HLA stabilization and KIR binding assay

HLA stabilization and KIR binding assays were performed as previously described [16]. In short, cells were washed with FBS free RPMI 1640 (R0) to remove any remaining foreign peptides from culturing in R10. Afterwards 721.221-ICP47-C*03:04, or 221-C*03:04-*TAP1*-KO cells were pulsed with 100 μM of the respective HCV peptide for 20 h at 26 °C. Previously described peptides that stabilized HLA-C*03:04 expression GAVDPLLAL (GAL) and GAVDPLLKL (GKL) [17], were used as positive controls, whereas culturing in the absence of peptides was used as negative control. After peptide-pulsing, cells were stained using an anti-HLA-ABC antibody (clone W6/32), fixed in 4% paraformaldehyde and analyzed using flow cytometry. KIR binding was analyzed after 20 h of HLA stabilization, as described above, with the respective peptides of interest. Cells were stained using KIR2DL3-Fc chimera (R&D), for 1 h on ice, secondary staining with anti-human FC antibody was performed for 30 min on ice. Afterwards cells were fixed and analyzed using flow cytometry.

NK cell degranulation assay

Primary NK cells were isolated from PBMCs of healthy donors, using Ficoll-Hypaque centrifugation, and rested overnight in R10 supplemented with 1 ng/ml IL-15. NK cells were co-incubated with peptide-pulsed 721.221-ICP47-C*03:04 cells at an effector to target (E:T) ratio of 5:1 in the presence of 3 μl anti-CD107a in 96 well plates. After 1 h incubation at 37 °C, GolgiPlug™ (BD) was added followed by 5 additional hours of incubation at 26 °C. Cells were subsequently stained using anti-CD3, anti-CD16, anti-CD56 and anti-KIR2DL3 for 30 min at 4 °C, fixed using 4% paraformaldehyde in PBS for 30 min and analyzed by flow cytometry. The gating strategy to identify responses in $KIR2DL3^+$ and $KIR2DL3^-$ cells is shown in Supplementary Fig. 1.

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.). Figures were designed and statistical analysis done using GraphPad Prism 5 (GraphPad Software, Inc.). All values in bar graphs represent mean ± SEM unless stated otherwise. Association of KIR2DL3 with HCV genotype was performed in GraphPad using the latest metadata [18] and data on KIR distribution in different ethnicities, kindly provided by Mary Carrington.

Results

*Several peptides derived from the core protein and non-structural protein 3 of HCV bind to HLA-C*03:04*

The goal of this study was to investigate the effect of HCV core and non-structural protein 3 (NS3)-derived epitopes presented by HLA-C*03:04 on binding to KIR2DL3 and modulation of NK cell activation. To identify suitable epitopes that are presented by HLA-C*03:04, we screened a pool of 200 overlapping peptides (OLP) of 15 amino acid length and overlapping by 11 amino acids, spanning both core and NS3 of HCV genotype 1. We identified 31 peptides that stabilized HLA-C*03:04 (Fig. 1) on the surface of 721.221-ICP47-C*03:04 cells. We subsequently focused on those peptides that showed a clear increase in HLA-C*03:04 expression, defined by having a MFI higher than the mean value plus 2 standard deviations of all non-stabilizing peptides for further assessment, resulting in ten 15mer peptides (Fig. 1 and Supplementary Fig. 2).

As the optimal length of a peptide binding to HLA class I molecules is between 9–11 amino acids, we attempted to predict the optimal binding sequence for HLA-C*03:04 within these ten 15mer peptides, based on previously published data and binding motifs (Table 1). These 9mer peptides were compared to the original 15mer peptides for their ability to stabilize HLA-C*03:04 expression (Table 1).

Two of the newly synthesized 9mer peptides, 34* and 120*, showed a markedly higher stabilization of HLA-C*03:04 compared to the original 15mer, while the other 9mer peptides showed no improved or even reduced HLA stabilization capacity. These two 9mer peptides and the original ten 15mer peptides were subsequently used to investigate their impact on the binding of KIR2DL3 to the HLA-peptide complex. We performed titration experiments to identify the optimal concentration to be used in later assays (Supplementary Fig. 4). Taken together, we identified 10 novel HCV peptides that bound to HLA-C*03:04.

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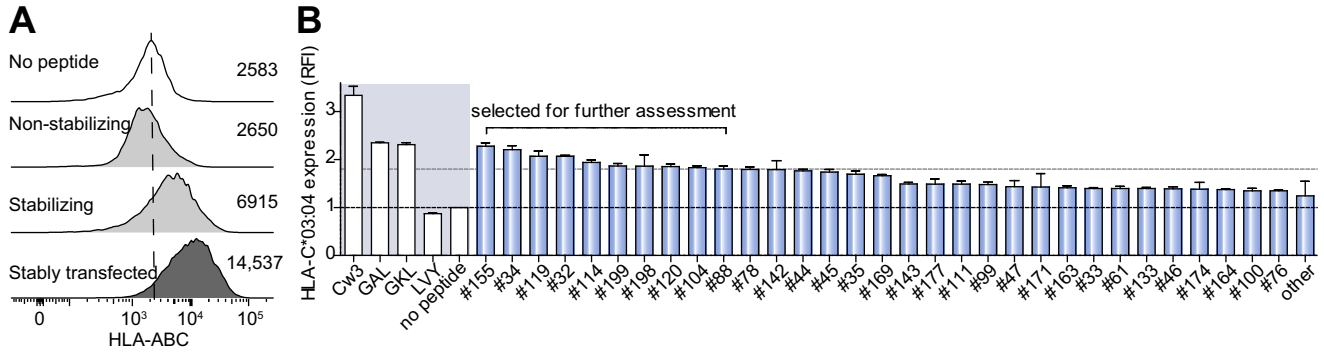

Fig. 1. HCV core and NS3 derived peptides stabilize HLA-C*03:04. (A) Histograms of HLA-ABC staining of 721.221-ICP47-C*03:04 cells pulsed with control peptides. The numbers represent the MFI values obtained during the measurement depicted. (B) HLA-C*03:04 expression on HCV core and NS3 peptide-pulsed 721.221-ICP47-C*03:04 cells. HLA-C*03:04 expression is shown as relative mean fluorescence intensity (RFI) as compared to unloaded 721.221-ICP47-C*03:04 cells. Each bar represents mean ± SEM of 3 independent experiments for each peptide. The black line represents the cutoff set by the no peptide control and the grey line the cutoff for the selected peptides. The grey area is underlying the positive and negative controls.

Table 1. HLA stabilization comparing 15mer and 9mer sequences.

15mer			9mer		
Number	Sequence	HLA stabilization	Number	Sequence	HLA stabilization
32	**TLTCGFADL**MGYIPL core$_{125-139}$	2.5	32*	**TLTCGFADL** core$_{125-133}$	1.0
34	LMG**YIPLVGAPL**GGA core$_{133-147}$	3.0	34*	**YIPLVGAPL** core$_{136-144}$	3.3
88	FI**PVENLETTM**RSPV NS3$_{1195-1209}$	2.4	88*	**PVENLETTM** NS3$_{1197-1205}$	1.0
104	**AATLGFGAY**MSKAHG NS3$_{1259-1273}$	2.7	104*	**AATLGFGAY** NS3$_{1259-1267}$	1.1
114	FLADGG**CSGGAYDII** NS3$_{1299-1313}$	2.5	114*	**CSGGAYDII** NS3$_{1305-1313}$	1.1
119	**HSTDATSIL**GIGTVL NS3$_{1319-1333}$	2.5	119*	**HSTDATSIL** NS3$_{1319-1327}$	1.6
120	AT**SILGIGTVL**DQAE NS3$_{1323-1337}$	2.3	120*	**SILGIGTVL** NS3$_{1325-1333}$	3.3
142	GI**NAVAYYRGL**DVSV NS3$_{1411-1425}$	1.8	142*	**NAVAYYRGL** NS3$_{1413-1421}$	1.6
155	D**FSLDPTFTI**ETITL NS3$_{1463-1477}$	3.1	155*	**FSLDPTFTI** NS3$_{1464-1472}$	1.2
198/199	****VTKYIMTCM**** NS3$_{1639-1653}$	2.4	198/199*	**VTKYIMTCM** NS3$_{1641-1649}$	0.9

*Binding of KIR2DL3 to HLA-C*03:04 is significantly increased by one HCV core derived epitope which also inhibits KIR2DL3$^+$ NK cell function*

To analyze the effects of the twelve selected peptides on binding of KIR2DL3 to HLA-C*03:04$^+$ cells, we loaded 721.221-ICP47-C*03:04 cells with the respective peptides and assessed KIR binding using a KIR2DL3-IgG fusion construct (Fig. 2A). The most dramatic increase in KIR2DL3-binding was observed for the 9mer peptide number 34* (YIPLVGAPL, referred to as YLVL), spanning position 136-144 of the core protein of HCV genotype 1 (Fig. 2B and Supplementary Fig. 3). The longer corresponding 15mer peptide number 34 (LMGYIPLVGAPLGGA), exhibited the second highest KIR binding. While some of the other peptides also allowed for some KIR2DL3 binding, binding was lower compared to YLVL. Titration experiments performed to determine the optimal concentration of YLVL 34* revealed similar concentration dependent kinetics as HLA stabilization (Supplementary Fig. 4).

We next addressed the functional consequence of the increased engagement of KIR2DL3 by YLVL presented by HLA-C*03:04 on KIR2DL3$^+$ NK cell function. For this we performed degranulation assays using purified primary NK cells from healthy donors in combination with YLVL-pulsed 721.221-ICP47-C*03:04 cells as target cells (Fig. 3A). The above observed strong binding of KIR2DL3 to the HLA-C*03:04$^+$ cells pulsed with YLVL peptide also resulted in a significant reduction in primary NK cell responses towards the target cells (Fig. 3B). This reduction in NK cell responses, while barely visible for the whole CD56^{dim} NK cell population, was dramatically increased in the KIR2DL3$^+$ NK cell subset. In contrast, primary NK cells lacking KIR2DL3 were much less affected by the peptide-pulsed target cells. Taken together, these data demonstrate that the newly identified HCV

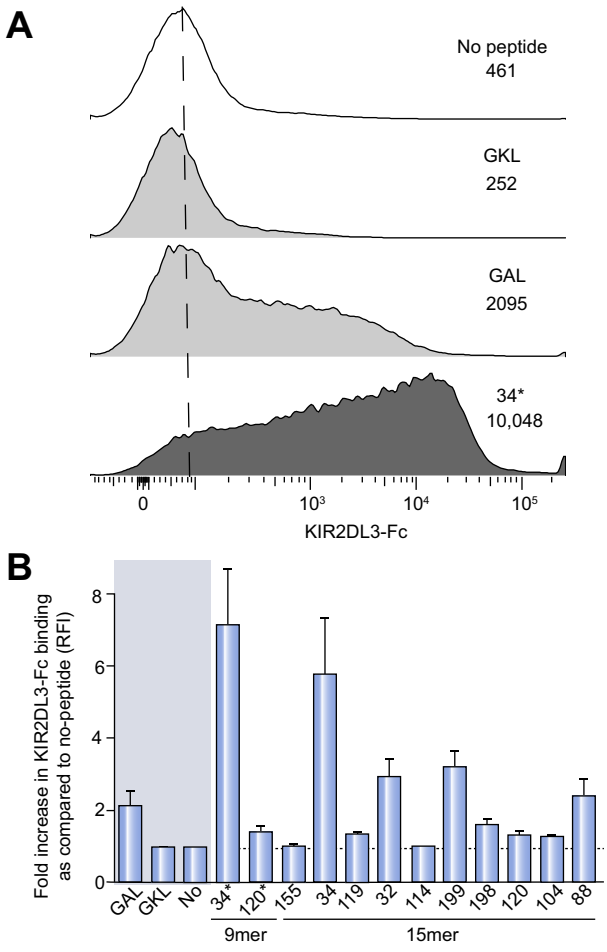

Fig. 2. Binding of KIR2DL3-Fc to HCV core and NS3 derived peptides presented by HLA-C*03:04. (A) Representative KIR2DL3-Fc staining on peptide pulsed 721.221-ICP47-C*03:04 cells. Histograms show no peptide control (white), a non-binding peptide (upper grey), a binding peptide (lower grey) and peptide 34* (black). The numbers represent the MFI values obtained during the measurement depicted. (B) Relative binding of KIR2DL3-Fc to HCV core and NS3 derived peptide-pulsed 721.221-ICP47-C*03:04 cells. KIR2DL3-Fc binding is shown as relative mean fluorescence intensity (RFI) compared to unloaded 721.221-ICP47-C*03:04 cells. Each bar represents mean ± SEM of 3 independent experiments for each peptide. The grey area is underlying the positive and negative controls.

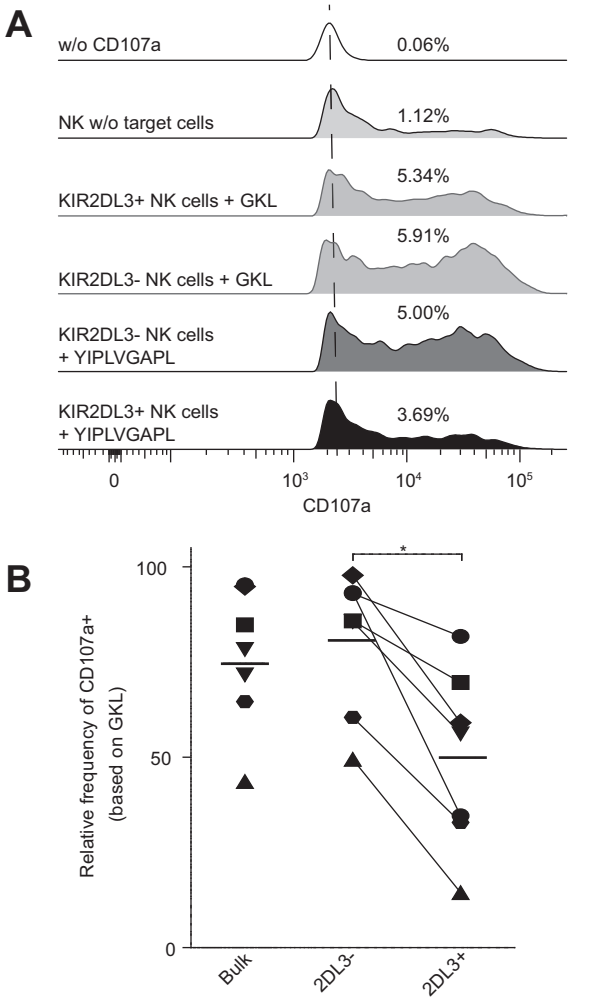

Fig. 3. YLVL peptide inhibits primary NK cell function when presented on HLA-C*03:04 cells. (A) Representative staining of CD107a on NK cells after co-incubation with peptide pulsed 721.221-ICP47-C*03:04. Control without CD107a-AB, NK cells without target cells, KIR2DL3− and KIR2DL3+ NK cells against YLVL pulsed target cells. The numbers represent the % of CD107a$^+$ NK cells for the depicted measurement. (B) Relative frequency of CD107a$^+$ NK cells when co-cultured with 721.221-ICP47-C*03:04 cells pulsed with YLVL compared to GKL control. Each bar represents mean ± SEM of 7 independent experiments for each peptide.

peptide YIPLVGAPL inhibits KIR2DL3$^+$ NK cell function by increasing the binding of KIR2DL3 to HLA-C*03:04.

Naturally occurring sequence variations of YIPLVGAPL modulate KIR2DL3 binding and NK cell function

The peptides we used for our initial screening assays were based on the HCV genotype 1 sequence. Comparison with other HCV genotypes revealed that the YLVL peptide often contained sequence variations in other HCV genotypes. We next assessed whether these sequence variations have a differential effect on HLA-C*03:04 stabilization, KIR2DL3 binding and KIR2DL3$^+$ NK cell function. In other genotypes, the leucine in position 4 and 9 of **Y**IP**LV**GAP**L** often changes to valine, resulting in the following peptide variants; **Y**IP**VV**GAP**L**, **Y**IP**LV**GAP**V** or **Y**IP**VV**GAP**V** (Table 2). The variants for HCV genotype 6, 3/4 and 2 in combination with genotype 1 accounted for the four most common variants, which amount to 88.7% of all published sequences for HCV core$_{136\text{-}144}$ (hcv.lanl.gov).

We therefore assessed whether these naturally occurring sequence variations might show a differential effect on KIR2DL3 binding and subsequent activation of KIR2DL3$^+$ NK cells. 721.221-ICP47-C*03:04 cells were pulsed with peptides corresponding to the different variations of core$_{136\text{-}144}$ and HLA-C*03:04 stabilization and KIR2DL3 binding were assessed (Fig. 4A, B). The genotype 2 (YVVV) variant peptide of core$_{136\text{-}144}$ showed a markedly decreased ability to stabilize HLA-C*03:04 expression compared to the genotype 1 YLVL peptide, while the other variant peptides exhibited similar stabilization capacities. Overall the genotype 1 derived peptide YLVL showed the highest capability to increase KIR2DL3 binding compared to YLVV and YVVV, but not the YVVL sequence. The different capacity of the four variant peptides to increase binding of KIR2DL3 to

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Table 2. Naturally occurring sequence variations in HCV core$_{136-144}$.

Name	Main HCV genotype	Sequence	Frequency of HCV sequences
YLVL	1	**YIPLV**GAP**L**	69.5%
YVVL	6	**YIPVV**GAP**L**	7.7%
YLVV	3 + 4	**YIPLV**GAP**V**	6.0%
YVVV	2	**YIPVV**GAP**V**	5.5%

peptide-pulsed 721.221-ICP47-C*03:04 cells was mirrored in their ability to modulate NK cell function. Functional responses of KIR2DL3$^+$ NK cells were generally lower for those viral variant peptides that showed higher KIR2DL3 binding and HLA stabilization, with YLVL showing the overall highest inhibition followed by YVVL, YLVV and finally YVVV.

Overall we observed a modulation of KIR2DL3$^+$ NK cell function by naturally occurring variants of the core$_{136-144}$ peptide based on the ability of these peptide variants to stabilize HLA-C*03:04 expression as well as the capacity to increase binding of KIR2DL3 to peptide-pulsed 721.221-ICP47-C*03:04 cells.

Discussion

Understanding the complexity of host-pathogen interactions involving multiple arms of the immune system remains one of the major challenges in immunology. Prompted by observations that KIR-HLA interactions play an important role in the clearance of HCV infection [12,14] and that NK cells can impose immune selection pressure in several viral infections [1], we investigated the impact of HCV-derived peptides on KIR-HLA interactions and primary NK cell function. We identified several novel HLA-C*03:04-binding epitopes in HCV core and NS3, and show that the HLA-C*03:04-restricted peptide YIPLVGAPL derived from the core protein (position 136-144) of HCV genotype 1 can inhibit KIR2DL3$^+$ NK cell function. We furthermore demonstrate that several naturally occurring sequence variations of YIPLVGAPL in other HCV genotypes exhibit reduced capacities to bind to the inhibitory NK cell receptor KIR2DL3 and a lower capacity to inhibit KIR2DL3$^+$ NK cell function. Taken together, these data provide a novel mechanism by which HCV might be able to evade NK cell-mediated recognition through the presentation of viral epitopes that engage inhibitory NK cell receptors.

HCV genotype 1 accounts for roughly 40–50% of all hepatitis C cases worldwide and remains a major health burden, even in light of the new highly effective DAA treatments [19,20]. Why 10–50% of all symptomatic patients are able to clear the infection spontaneously, while the rest develop persistent infections, remains unclear [21]. Recent data suggest that host genetic factors contribute importantly to this differential outcome of HCV infection and disease progression. The most prominent host gene polymorphisms associated to date with HCV disease outcome, *IL28B* [22] and *KIR2DL3* [12], both represent genes of the innate immune system. The protective effect of certain *IL28B* polymorphisms might be due to an increased ability of hepatocytes to respond to members of the interferon family [23] leading to an increased antiviral state in potential target cells. The described effect of KIR2DL3 and HLA-C alleles of the HLA-C group 1 (HLA-C1) family that serve as ligands for KIR2DL3, including HLA-C*03:04, on HCV clearance [12] is thought to be due to the comparatively weak interaction between HLA-C1 molecules and the inhibitory KIR2DL3 receptor [24]. This weak inhibitory interaction might result in stronger antiviral NK cell responses in HLA-C1/KIR2DL3$^+$ individuals, and a strengthening of the binding of KIR2DL3 to HLA-C1 molecules might provide a benefit to the virus. Here we show that the HCV core-derived viral epitope YIPLVGAPL can increase binding of inhibitory KIR2DL3 to the respective HLA-C*03:04/peptide complex, leading to a significant inhibition of KIR2DL3$^+$ NK cell function.

One of the limitations of our study is that we used peptides of 15AA length for the initial HLA stabilization screen. We therefore cannot exclude the possibility that we might have missed some peptides which also stabilize HLA-C*03:04. Nevertheless none

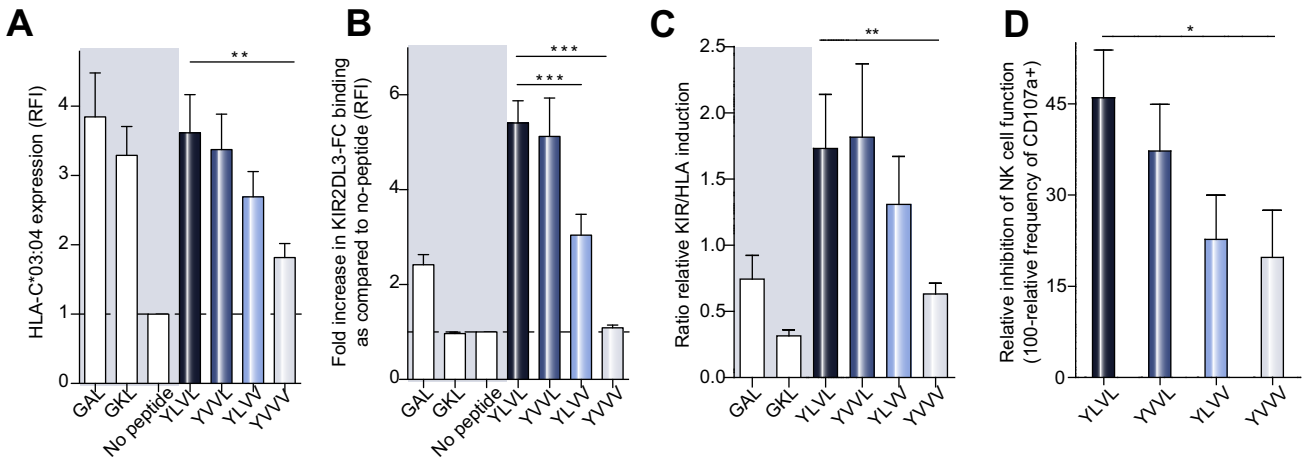

Fig. 4. Sequence-specific modulation of KIR2DL3 binding and NK cell function. (A) HLA-C*03:04 expression by 721.221-ICP47-C*03:04 cells pulsed with genotype variants of YLVL. (B) Relative binding of KIR2DL3-Fc to 721.221-ICP47-C*03:04 cells pulsed with genotype variants of YLVL. (C) Relative induction of KIR2DL3 binding in relation to stabilization of HLA-C*03:04 on 721.221-ICP47-C*03:04 cells pulsed with genotype variants of YLVL. (D) Relative frequency of CD107a$^+$ NK cells out of KIR2DL3$^+$ NK cells when co-cultured with 721.221-ICP47-C*03:04 cells pulsed with YLVL and its genotype variants in comparison to GKL. Each bar represents the mean ± SEM of 5 independent experiments. The grey area is underlying the positive and negative controls.

of the tested 9mer or 15mer peptides showed similar effects on KIR binding compared to YIPLVGAPL and the original 15mer P34.

Previous studies have demonstrated that the sequences of peptides presented by HLA class I molecules impact KIR binding and KIR$^+$ NK cell function [17], including self-epitopes as well as peptides derived from EBV [25,26] and HIV [16,27]. However, no data on the ability of HCV peptides to modulate HLA/KIR interactions have been reported to our knowledge to date, despite strong data showing an important role for KIR-HLA interactions in HCV disease outcome [12,13]. Our data demonstrate that most HLA-C*03:04-restricted HCV peptides identified did not allow for binding of KIR2DL3, but that one peptide, YIPLVGAPL derived from HCV core, induced strong binding of KIR2DL3 to HLA-C*03:04$^+$ target cells and inhibited the function of KIR2DL3$^+$ primary NK cells. The crystal structure of HLA-Cw3 with KIR2DL3 was described several years ago [28], and illustrated that role of residues in the HLA-presented epitope, in particular positions 7 and 8, in the binding to KIR2DL3. Interestingly, while the sequence of the YIPLVGAPL peptide does not follow the described canonical peptide sequence motive for HLA-C*03:04 [29], it nevertheless stabilized HLA-C*03:04 expression on TAP-deficient cell lines and increased the binding of KIR2DL3. This is in line with recent data demonstrating that the binding motives for peptide presented by HLA-C molecules allow for more variability in the peptide sequence [29]. One concern might be the levels of HLA expression on the generated cell lines compared to normal human cells. We did however not find higher levels of HLA expression, when comparing the generated cell lines pulsed with stabilizing peptides to human PBMCs (Supplementary Fig. 5).

A number of naturally occurring sequence variations of YIPLVGAPL are described; which are primarily seen in other HCV genotypes. These variations are characterized by a switch from leucine to valine at position 4 and/or 9, and showed effects on HLA stabilization, KIR binding and NK cell function in our studies. In particular, the switch from leucine to valine in the C-terminal HLA-binding anchor position 9 of the peptide resulted in reduction of HLA-C*03:04 expression on TAP-deficient cell lines, in line with the critical role of this residue in binding to HLA-C*03:04. The observed reduction in KIR2DL3 binding in the context of these two peptides (YIPLVGAPV, YIPVVGAPV) is most likely a reflection of the reduced expression of its ligand HLA-C*03:04. In contrast, changes in position 4 had little effect on HLA expression, KIR2DL3 binding or the function of KIR2DL3$^+$ NK cells. It remains unknown whether NK cell-mediated immune pressure could induce HCV to adapt to the KIR/HLA genotypes in certain host populations, exploiting the ability of specific sequence variants in HLA class I-presented epitopes to inhibit KIR+ NK cell function, as suggested in the context of HIV infection [10]. When we compared KIR distribution in different ethnicities with the latest meta-data analysis on HCV genotype distribution [18], we found that, among HCV infected individuals, carrying the KIR2DL3 gene strongly correlates ($R^2 = 0.8317$, $p < 0.0001$) with being infected with HCV genotype 1 (data not shown). Future studies are also required to determine the abundance by which the identified HCV peptides are presented by HLA class I on HCV infected cells *in vivo*, and whether the function of intrahepatic NK cells is modified by HLA class I-presented HCV peptides to the same degree, as a recent publication hinted towards distinct functional features of intrahepatic NK cells [30].

In conclusion we identified several novel HLA-C*03:04-presented epitopes in HCV core and NS3, and provide novel functional evidence demonstrating that one of these HCV-derived peptides presented by HLA-C*03:04 can inhibit primary NK cell function through the engagement of an inhibitory KIR. These data provide first evidence for a novel pathway by which HCV can inhibit recognition by NK cells through the engagement of inhibitory NK cell receptors, providing a possible mechanism for viral escape.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Authors' contributions

SL designed and performed the experiments, analyzed the data and wrote the paper; GM performed experiments and wrote the paper; AH designed the experiments and wrote the paper; AC and MZ performed experiments; CK and WGM designed experiments and wrote the paper; MC analyzed the data and wrote the paper; HW analyzed the data and wrote the paper; MA designed the experiments, analyzed the data and wrote the paper.

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Supplementary data

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Author names in bold designate shared co-first authorship

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Other

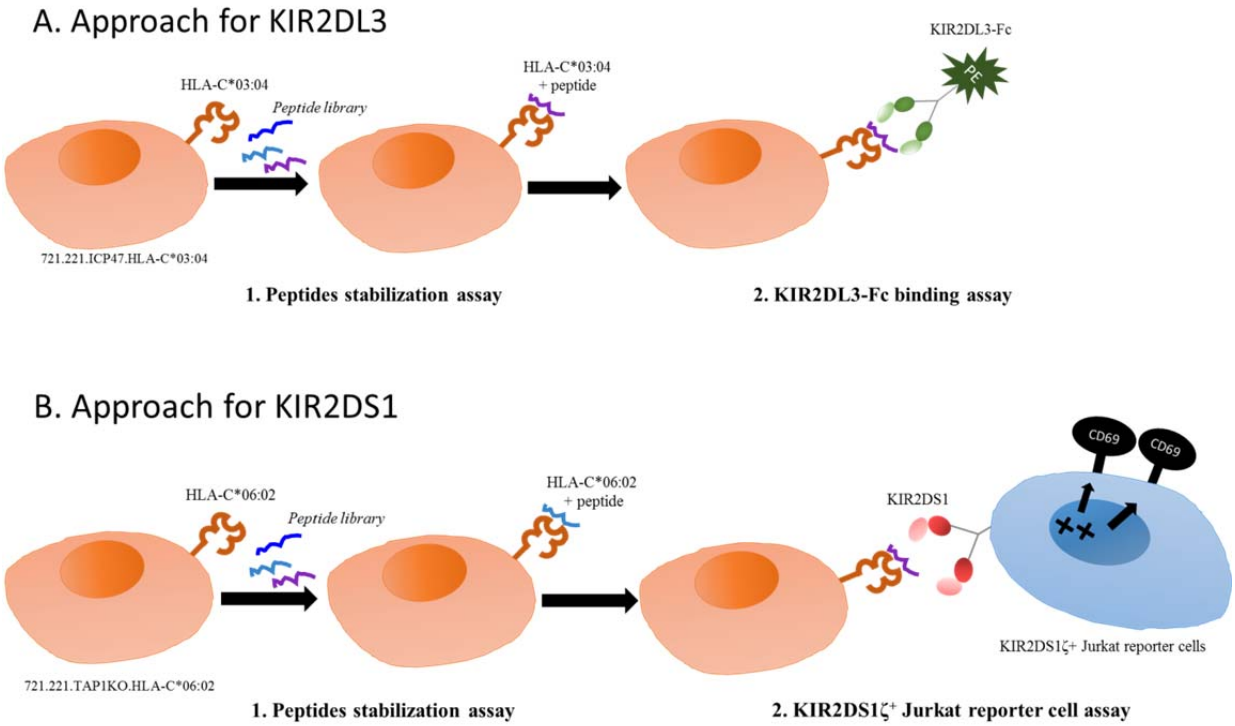

Fig 1. KIR cellular reporter system

This model consist of HLA class I deficient B cells lines named 721.221 transduced with one HLA-C molecule, respectively HLA-C*03:04 or HLA-C*06:02. To prevent the cell lines to present self-peptides onto their respective HLA-C molecules, loading of endogenous peptides was disabled using two experimental strategies. First, 721.221.HLA-C*03:04 cell line was transfected with ICP47, an herpex simplex virus type 1 (HSV-1) protein known to block TAP, a molecule involved in the loading of the peptide to the HLA class I molecule, and the subsequent cell line was named 721.221.ICP47.HLA-C*03:04. Second, TAP1 gene was deleted using CRISPR/CAS9 technology in the 721.221.HLA-C*06:02 cell line and consequently named 721.221.TAP1KO.HLA-C*06:02. The absence of endogenous peptides increases the turnover of HLA-C molecules which cannot be stably expressed at the cell surface. Therefore, the cell lines 721.221.ICP47.HLA-C*03:04 and 721.221.TAP1KO.HLA-C*06:02 can be pulsed with external, single candidate peptides. Strong binders' peptides are identified by their capacity to stabilize HLA-C expression. The selected peptides were further studied for their capacity to bind KIR2DL3 and KIR2DS1. For this purpose, two different experimental approaches were used (A) KIR2DL3-IgG Fusion Construct were used to screen 721.221.ICP47.HLA-C*03:04 cells pulsed with the peptide identified. A secondary PE-antibody directed against the IgG part of KIR2DL3 allowed to measure the intensity of KIR2DL3 binding by flow cytometry. (B) KIR2DS1ζ^+ expressing Jurkat reporter cells were produced and used to screen 721.221.TAP1KO.HLA-C*06:02 pulsed with the peptides identified. Binding of KIR2DS1 to one HLA-C*06:02/peptide complex would result in activation of the KIR2DS1ζ^+ Jurkat reporter cells resulting in an increase of the expression levels of CD69 activation marker at the surface of the cells. The increase of reporter cell activity was measure by flow cytometry.

Table 1. HCV peptide library (genotype 1, subtype 1).
The peptides highlighted in grey were sectioned for HLA-C*06:02 stabilizing assays.

Name	Type	Sequence
HCV-1	core	MSTNPKPQKKNKRNT
HCV-2	core	PKPQKKNKRNTNRRP
HCV-3	core	KKNKRNTNRRPQDVK
HCV-4	core	RNTNRRPQDVKFPGG
HCV-5	core	RRPQDVKFPGGGQIV
HCV-6	core	DVKFPGGGQIVGGVY
HCV-7	core	PGGGQIVGGVYLLPR
HCV-8	core	QIVGGVYLLPRRGPR
HCV-9	core	GVYLLPRRGPRLGVR
HCV-10	core	LPRRGPRLGVRATRK
HCV-11	core	GPRLGVRATRKTSER
HCV-12	core	GVRATRKTSERSQPR
HCV-13	core	TRKTSERSQPRGRRQ
HCV-14	core	SERSQPRGRRQPIPK
HCV-15	core	QPRGRRQPIPKARRP
HCV-16	core	RRQPIPKARRPEGRT
HCV-17	core	IPKARRPEGRTWAQP
HCV-18	core	RRPEGRTWAQPGYPW
HCV-19	core	GRTWAQPGYPWPLYG
HCV-20	core	AQPGYPWPLYGNEGC
HCV-21	core	YPWPLYGNEGCGWAG
HCV-22	core	LYGNEGCGWAGWLLS
HCV-23	core	EGCGWAGWLLSPRGS
HCV-24	core	WAGWLLSPRGSRPSW
HCV-25	core	LLSPRGSRPSWGPTD
HCV-26	core	RGSRPSWGPTDPRRR
HCV-27	core	PSWGPTDPRRRSRNL
HCV-28	core	PTDPRRRSRNLGKVI
HCV-29	core	RRRSRNLGKVIDTLT
HCV-30	core	RNLGKVIDTLTCGFA
HCV-31	core	KVIDTLTCGFADLMG
HCV-32	core	TLTCGFADLMGYIPL
HCV-33	core	GFADLMGYIPLVGAP
HCV-34	core	LMGYIPLVGAPLGGA
HCV-35	core	IPLVGAPLGGAARAL
HCV-36	core	GAPLGGAARALAHGV
HCV-37	core	GGAARALAHGVRVLE
HCV-38	core	RALAHGVRVLEDGVN
HCV-39	core	HGVRVLEDGVNYATG
HCV-40	core	VLEDGVNYATGNLPG
HCV-41	core	GVNYATGNLPGCSFS

HCV-42	core	ATGNLPGCSFSIFLL
HCV-43	core	LPGCSFSIFLLALLS
HCV-44	core	SFSIFLLALLSCLTV
HCV-45	core	FLLALLSCLTVPASA
HCV-46	NS3	APITAYAQQTRGLLG
HCV-47	NS3	AYAQQTRGLLGCIIT
HCV-48	NS3	QTRGLLGCIITSLTG
HCV-49	NS3	LLGCIITSLTGRDKN
HCV-50	NS3	IITSLTGRDKNQVEG
HCV-51	NS3	LTGRDKNQVEGEVQI
HCV-52	NS3	DKNQVEGEVQIVSTA
HCV-53	NS3	VEGEVQIVSTAAQTF
HCV-54	NS3	VQIVSTAAQTFLATC
HCV-55	NS3	STAAQTFLATCINGV
HCV-56	NS3	QTFLATCINGVCWTV
HCV-57	NS3	ATCINGVCWTVYHGA
HCV-58	NS3	NGVCWTVYHGAGTRT
HCV-59	NS3	WTVYHGAGTRTIASP
HCV-60	NS3	HGAGTRTIASPKGPV
HCV-61	NS3	TRTIASPKGPVIQMY
HCV-62	NS3	ASPKGPVIQMYTNVD
HCV-63	NS3	GPVIQMYTNVDQDLV
HCV-64	NS3	QMYTNVDQDLVGWPA
HCV-65	NS3	NVDQDLVGWPAPQGS
HCV-66	NS3	DLVGWPAPQGSRSLT
HCV-67	NS3	WPAPQGSRSLTPCTC
HCV-68	NS3	QGSRSLTPCTCGSSD
HCV-69	NS3	SLTPCTCGSSDLYLV
HCV-70	NS3	CTCGSSDLYLVTRHA
HCV-71	NS3	SSDLYLVTRHADVIP
HCV-72	NS3	YLVTRHADVIPVRRR
HCV-73	NS3	RHADVIPVRRRGDSR
HCV-74	NS3	VIPVRRRGDSRGSLL
HCV-75	NS3	RRRGDSRGSLLSPRP
HCV-76	NS3	DSRGSLLSPRPISYL
HCV-77	NS3	SLLSPRPISYLKGSS
HCV-78	NS3	PRPISYLKGSSGGPL
HCV-79	NS3	SYLKGSSGGPLLCPA
HCV-80	NS3	GSSGGPLLCPAGHAV
HCV-81	NS3	GPLLCPAGHAVGIFR
HCV-82	NS3	CPAGHAVGIFRAAVC
HCV-83	NS3	HAVGIFRAAVCTRGV
HCV-84	NS3	IFRAAVCTRGVAKAV
HCV-85	NS3	AVCTRGVAKAVDFIP

HCV-86	NS3	RGVAKAVDFIPVENL
HCV-87	NS3	KAVDFIPVENLETTM
HCV-88	NS3	FIPVENLETTMRSPV
HCV-89	NS3	ENLETTMRSPVFTDN
HCV-90	NS3	TTMRSPVFTDNSSPP
HCV-91	NS3	SPVFTDNSSPPVVPQ
HCV-92	NS3	TDNSSPPVVPQSFQV
HCV-93	NS3	SPPVVPQSFQVAHLH
HCV-94	NS3	VPQSFQVAHLHAPTG
HCV-95	NS3	FQVAHLHAPTGSGKS
HCV-96	NS3	HLHAPTGSGKSTKVP
HCV-97	NS3	PTGSGKSTKVPAAYA
HCV-98	NS3	GKSTKVPAAYAAQGY
HCV-99	NS3	KVPAAYAAQGYKVLV
HCV-100	NS3	AYAAQGYKVLVLNPS
HCV-101	NS3	QGYKVLVLNPSVAAT
HCV-102	NS3	VLVLNPSVAATLGFG
HCV-103	NS3	NPSVAATLGFGAYMS
HCV-104	NS3	AATLGFGAYMSKAHG
HCV-105	NS3	GFGAYMSKAHGIDPN
HCV-106	NS3	YMSKAHGIDPNIRTG
HCV-107	NS3	AHGIDPNIRTGVRTI
HCV-108	NS3	DPNIRTGVRTITTGS
HCV-109	NS3	RTGVRTITTGSPITY
HCV-110	NS3	RTITTGSPITYSTYG
HCV-111	NS3	TGSPITYSTYGKFLA
HCV-112	NS3	ITYSTYGKFLADGGC
HCV-113	NS3	TYGKFLADGGCSGGA
HCV-114	NS3	FLADGGCSGGAYDII
HCV-115	NS3	GGCSGGAYDIIICDE
HCV-116	NS3	GGAYDIIICDECHST
HCV-117	NS3	DIIICDECHSTDATS
HCV-118	NS3	CDECHSTDATSILGI
HCV-119	NS3	HSTDATSILGIGTVL
HCV-120	NS3	ATSILGIGTVLDQAE
HCV-121	NS3	LGIGTVLDQAETAGA
HCV-122	NS3	TVLDQAETAGARLVV
HCV-123	NS3	QAETAGARLVVLATA
HCV-124	NS3	AGARLVVLATATPPG
HCV-125	NS3	LVVLATATPPGSVTV
HCV-126	NS3	ATATPPGSVTVPHPN
HCV-127	NS3	PPGSVTVPHPNIEEV
HCV-128	NS3	VTVPHPNIEEVALST
HCV-129	NS3	HPNIEEVALSTTGEI

HCV-130	NS3	EEVALSTTGEIPFYG
HCV-131	NS3	LSTTGEIPFYGKAIP
HCV-132	NS3	GEIPFYGKAIPLEVI
HCV-133	NS3	FYGKAIPLEVIKGGR
HCV-134	NS3	AIPLEVIKGGRHLIF
HCV-135	NS3	EVIKGGRHLIFCHSK
HCV-136	NS3	GGRHLIFCHSKKKCD
HCV-137	NS3	LIFCHSKKKCDELAA
HCV-138	NS3	HSKKKCDELAAKLVA
HCV-139	NS3	KCDELAAKLVALGIN
HCV-140	NS3	LAAKLVALGINAVAY
HCV-141	NS3	LVALGINAVAYYRGL
HCV-142	NS3	GINAVAYYRGLDVSV
HCV-143	NS3	VAYYRGLDVSVIPTS
HCV-144	NS3	RGLDVSVIPTSGDVV
HCV-145	NS3	VSVIPTSGDVVVVAT
HCV-146	NS3	PTSGDVVVVATDALM
HCV-147	NS3	DVVVVATDALMTGYT
HCV-148	NS3	VATDALMTGYTGDFD
HCV-149	NS3	ALMTGYTGDFDSVID
HCV-150	NS3	GYTGDFDSVIDCNTC
HCV-151	NS3	DFDSVIDCNTCVTQT
HCV-152	NS3	VIDCNTCVTQTVDFS
HCV-153	NS3	NTCVTQTVDFSLDPT
HCV-154	NS3	TQTVDFSLDPTFTIE
HCV-155	NS3	DFSLDPTFTIETITL
HCV-156	NS3	DPTFTIETITLPQDA
HCV-157	NS3	TIETITLPQDAVSRT
HCV-158	NS3	ITLPQDAVSRTQRRG
HCV-159	NS3	QDAVSRTQRRGRTGR
HCV-160	NS3	SRTQRRGRTGRGKPG
HCV-161	NS3	RRGRTGRGKPGIYRF
HCV-162	NS3	TGRGKPGIYRFVAPG
HCV-163	NS3	KPGIYRFVAPGERPS
HCV-164	NS3	YRFVAPGERPSGMFD
HCV-165	NS3	APGERPSGMFDSSVL
HCV-166	NS3	RPSGMFDSSVLCECY
HCV-167	NS3	MFDSSVLCECYDAGC
HCV-168	NS3	SVLCECYDAGCAWYE
HCV-169	NS3	ECYDAGCAWYELTPA
HCV-170	NS3	AGCAWYELTPAETTV
HCV-171	NS3	WYELTPAETTVRLRA
HCV-172	NS3	TPAETTVRLRAYMNT
HCV-173	NS3	TTVRLRAYMNTPGLP

HCV-174	NS3	LRAYMNTPGLPVCQD
HCV-175	NS3	MNTPGLPVCQDHLEF
HCV-176	NS3	GLPVCQDHLEFWEGV
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HCV-192	NS3	LIRLKPTLHGPTPLL
HCV-193	NS3	KPTLHGPTPLLYRLG
HCV-194	NS3	HGPTPLLYRLGAVQN
HCV-195	NS3	PLLYRLGAVQNEITL
HCV-196	NS3	RLGAVQNEITLTHPV
HCV-197	NS3	VQNEITLTHPVTKYI
HCV-198	NS3	ITLTHPVTKYIMTCM
HCV-199	NS3	HPVTKYIMTCMSADL
HCV-200	NS3	KYIMTCMSADLEVVT

Table 2. HIV-1 peptide library (HIV-1 consensus sequence, clade B)

Name	Type	Sequence
HIV-1	GAG p24	PIVQNLQGQM
HIV-2	GAG p24	IVQNLQGQMV
HIV-3	GAG p24	VQNLQGQMVH
HIV-4	GAG p24	QNLQGQMVHQ
HIV-5	GAG p24	NLQGQMVHQA
HIV-6	GAG p24	LQGQMVHQAI
HIV-7	GAG p24	QGQMVHQAIS
HIV-8	GAG p24	GQMVHQAISP
HIV-9	GAG p24	QMVHQAISPR
HIV-10	GAG p24	MVHQAISPRT
HIV-11	GAG p24	VHQAISPRTL
HIV-12	GAG p24	HQAISPRTLN
HIV-13	GAG p24	QAISPRTLNA
HIV-14	GAG p24	AISPRTLNAW
HIV-15	GAG p24	ISPRTLNAWV
HIV-16	GAG p24	SPRTLNAWVK
HIV-17	GAG p24	PRTLNAWVKV
HIV-18	GAG p24	RTLNAWVKVV
HIV-19	GAG p24	TLNAWVKVVE
HIV-20	GAG p24	LNAWVKVVEE
HIV-21	GAG p24	NAWVKVVEEK
HIV-22	GAG p24	AWVKVVEEKA
HIV-23	GAG p24	WVKVVEEKAF
HIV-24	GAG p24	VKVVEEKAFS
HIV-25	GAG p24	KVVEEKAFSP
HIV-26	GAG p24	VVEEKAFSPE
HIV-27	GAG p24	VEEKAFSPEV
HIV-28	GAG p24	EEKAFSPEVI
HIV-29	GAG p24	EKAFSPEVIP
HIV-30	GAG p24	KAFSPEVIPM
HIV-31	GAG p24	AFSPEVIPMF
HIV-32	GAG p24	FSPEVIPMFS
HIV-33	GAG p24	SPEVIPMFSA
HIV-34	GAG p24	PEVIPMFSAL
HIV-35	GAG p24	EVIPMFSALS
HIV-36	GAG p24	VIPMFSALSE
HIV-37	GAG p24	IPMFSALSEG
HIV-38	GAG p24	PMFSALSEGA
HIV-39	GAG p24	MFSALSEGAT
HIV-40	GAG p24	FSALSEGATP
HIV-41	GAG p24	SALSEGATPQ

HIV-42	GAG p24	ALSEGATPQD
HIV-43	GAG p24	LSEGATPQDL
HIV-44	GAG p24	SEGATPQDLN
HIV-45	GAG p24	EGATPQDLNT
HIV-46	GAG p24	GATPQDLNTM
HIV-47	GAG p24	ATPQDLNTML
HIV-48	GAG p24	TPQDLNTMLN
HIV-49	GAG p24	PQDLNTMLNT
HIV-50	GAG p24	QDLNTMLNTV
HIV-51	GAG p24	DLNTMLNTVG
HIV-52	GAG p24	LNTMLNTVGG
HIV-53	GAG p24	NTMLNTVGGH
HIV-54	GAG p24	TMLNTVGGHQ
HIV-55	GAG p24	MLNTVGGHQA
HIV-56	GAG p24	LNTVGGHQAA
HIV-57	GAG p24	NTVGGHQAAM
HIV-58	GAG p24	TVGGHQAAMQ
HIV-59	GAG p24	VGGHQAAMQM
HIV-60	GAG p24	GGHQAAMQML
HIV-61	GAG p24	GHQAAMQMLK
HIV-62	GAG p24	HQAAMQMLKE
HIV-63	GAG p24	QAAMQMLKET
HIV-64	GAG p24	AAMQMLKETI
HIV-65	GAG p24	AMQMLKETIN
HIV-66	GAG p24	MQMLKETINE
HIV-67	GAG p24	QMLKETINEE
HIV-68	GAG p24	MLKETINEEA
HIV-69	GAG p24	LKETINEEAA
HIV-70	GAG p24	KETINEEAAE
HIV-71	GAG p24	ETINEEAAEW
HIV-72	GAG p24	TINEEAAEWD
HIV-73	GAG p24	INEEAAEWDR
HIV-74	GAG p24	NEEAAEWDRL
HIV-75	GAG p24	EEAAEWDRLH
HIV-76	GAG p24	EAAEWDRLHP
HIV-77	GAG p24	AAEWDRLHPV
HIV-78	GAG p24	AEWDRLHPVH
HIV-79	GAG p24	EWDRLHPVHA
HIV-80	GAG p24	WDRLHPVHAG
HIV-81	GAG p24	DRLHPVHAGP
HIV-82	GAG p24	RLHPVHAGPI
HIV-83	GAG p24	LHPVHAGPIA
HIV-84	GAG p24	HPVHAGPIAP
HIV-85	GAG p24	PVHAGPIAPG

HIV-86	GAG p24	VHAGPIAPGQ
HIV-87	GAG p24	HAGPIAPGQM
HIV-88	GAG p24	AGPIAPGQMR
HIV-89	GAG p24	GPIAPGQMRE
HIV-90	GAG p24	PIAPGQMREP
HIV-91	GAG p24	IAPGQMREPR
HIV-92	GAG p24	APGQMREPRG
HIV-93	GAG p24	PGQMREPRGS
HIV-94	GAG p24	GQMREPRGSD
HIV-95	GAG p24	QMREPRGSDI
HIV-96	GAG p24	MREPRGSDIA
HIV-97	GAG p24	REPRGSDIAG
HIV-98	GAG p24	EPRGSDIAGT
HIV-99	GAG p24	PRGSDIAGTT
HIV-100	GAG p24	RGSDIAGTTS
HIV-101	GAG p24	GSDIAGTTST
HIV-102	GAG p24	SDIAGTTSTL
HIV-103	GAG p24	DIAGTTSTLQ
HIV-104	GAG p24	IAGTTSTLQE
HIV-105	GAG p24	AGTTSTLQEQ
HIV-106	GAG p24	GTTSTLQEQI
HIV-107	GAG p24	TTSTLQEQIG
HIV-108	GAG p24	TSTLQEQIGW
HIV-109	GAG p24	STLQEQIGWM
HIV-110	GAG p24	TLQEQIGWMT
HIV-111	GAG p24	LQEQIGWMTN
HIV-112	GAG p24	QEQIGWMTNN
HIV-113	GAG p24	EQIGWMTNNP
HIV-114	GAG p24	QIGWMTNNPP
HIV-115	GAG p24	IGWMTNNPPI
HIV-116	GAG p24	GWMTNNPPIP
HIV-117	GAG p24	WMTNNPPIPV
HIV-118	GAG p24	MTNNPPIPVG
HIV-119	GAG p24	TNNPPIPVGE
HIV-120	GAG p24	NNPPIPVGEI
HIV-121	GAG p24	NPPIPVGEIY
HIV-122	GAG p24	PPIPVGEIYK
HIV-123	GAG p24	PIPVGEIYKR
HIV-124	GAG p24	IPVGEIYKRW
HIV-125	GAG p24	PVGEIYKRWI
HIV-126	GAG p24	VGEIYKRWII
HIV-127	GAG p24	GEIYKRWIIL
HIV-128	GAG p24	EIYKRWIILG
HIV-129	GAG p24	IYKRWIILGL

HIV-130	GAG p24	YKRWIILGLN
HIV-131	GAG p24	KRWIILGLNK
HIV-132	GAG p24	RWIILGLNKI
HIV-133	GAG p24	WIILGLNKIV
HIV-134	GAG p24	IILGLNKIVR
HIV-135	GAG p24	ILGLNKIVRM
HIV-136	GAG p24	LGLNKIVRMY
HIV-137	GAG p24	GLNKIVRMYS
HIV-138	GAG p24	LNKIVRMYSP
HIV-139	GAG p24	NKIVRMYSPT
HIV-140	GAG p24	KIVRMYSPTS
HIV-141	GAG p24	IVRMYSPTSI
HIV-142	GAG p24	VRMYSPTSIL
HIV-143	GAG p24	RMYSPTSILD
HIV-144	GAG p24	MYSPTSILDI
HIV-145	GAG p24	YSPTSILDIR
HIV-146	GAG p24	SPTSILDIRQ
HIV-147	GAG p24	PTSILDIRQG
HIV-148	GAG p24	TSILDIRQGP
HIV-149	GAG p24	SILDIRQGPK
HIV-150	GAG p24	ILDIRQGPKE
HIV-151	GAG p24	LDIRQGPKEP
HIV-152	GAG p24	DIRQGPKEPF
HIV-153	GAG p24	IRQGPKEPFR
HIV-154	GAG p24	RQGPKEPFRD
HIV-155	GAG p24	QGPKEPFRDY
HIV-156	GAG p24	GPKEPFRDYV
HIV-157	GAG p24	PKEPFRDYVD
HIV-158	GAG p24	KEPFRDYVDR
HIV-159	GAG p24	EPFRDYVDRF
HIV-160	GAG p24	PFRDYVDRFY
HIV-161	GAG p24	FRDYVDRFYK
HIV-162	GAG p24	RDYVDRFYKT
HIV-163	GAG p24	DYVDRFYKTL
HIV-164	GAG p24	YVDRFYKTLR
HIV-165	GAG p24	VDRFYKTLRA
HIV-166	GAG p24	DRFYKTLRAE
HIV-167	GAG p24	RFYKTLRAEQ
HIV-168	GAG p24	FYKTLRAEQA
HIV-169	GAG p24	YKTLRAEQAS
HIV-170	GAG p24	KTLRAEQASQ
HIV-171	GAG p24	TLRAEQASQE
HIV-172	GAG p24	LRAEQASQEV
HIV-173	GAG p24	RAEQASQEVK

HIV-174	GAG p24	AEQASQEVKN
HIV-175	GAG p24	EQASQEVKNW
HIV-176	GAG p24	QASQEVKNWM
HIV-177	GAG p24	ASQEVKNWMT
HIV-178	GAG p24	SQEVKNWMTE
HIV-179	GAG p24	QEVKNWMTET
HIV-180	GAG p24	EVKNWMTETL
HIV-181	GAG p24	VKNWMTETLL
HIV-182	GAG p24	KNWMTETLLV
HIV-183	GAG p24	NWMTETLLVQ
HIV-184	GAG p24	WMTETLLVQN
HIV-185	GAG p24	MTETLLVQNA
HIV-186	GAG p24	TETLLVQNAN
HIV-187	GAG p24	ETLLVQNANP
HIV-188	GAG p24	TLLVQNANPD
HIV-189	GAG p24	LLVQNANPDC
HIV-190	GAG p24	LVQNANPDCK
HIV-191	GAG p24	VQNANPDCKT
HIV-192	GAG p24	QNANPDCKTI
HIV-193	GAG p24	NANPDCKTIL
HIV-194	GAG p24	ANPDCKTILK
HIV-195	GAG p24	NPDCKTILKA
HIV-196	GAG p24	PDCKTILKAL
HIV-197	GAG p24	DCKTILKALG
HIV-198	GAG p24	CKTILKALGP
HIV-199	GAG p24	KTILKALGPA
HIV-200	GAG p24	TILKALGPAA
HIV-201	GAG p24	ILKALGPAAT
HIV-202	GAG p24	LKALGPAATL
HIV-203	GAG p24	KALGPAATLE
HIV-204	GAG p24	ALGPAATLEE
HIV-205	GAG p24	LGPAATLEEM
HIV-206	GAG p24	GPAATLEEMM
HIV-207	GAG p24	PAATLEEMMT
HIV-208	GAG p24	AATLEEMMTA
HIV-209	GAG p24	ATLEEMMTAC
HIV-210	GAG p24	TLEEMMTACQ
HIV-211	GAG p24	LEEMMTACQG
HIV-212	GAG p24	EEMMTACQGV
HIV-213	GAG p24	EMMTACQGVG
HIV-214	GAG p24	MMTACQGVGG
HIV-215	GAG p24	MTACQGVGGP
HIV-216	GAG p24	TACQGVGGPG
HIV-217	GAG p24	ACQGVGGPGH

HIV-218	GAG p24	CQGVGGPGHK
HIV-219	GAG p24	QGVGGPGHKA
HIV-220	GAG p24	GVGGPGHKAR
HIV-221	GAG p24	VGGPGHKARV
HIV-222	GAG p24	GGPGHKARVL
HIV-223	Nef	MGGKWSKRSVVGWPAVR
HIV-224	Nef	RSVVGWPAVRERMRRA
HIV-225	Nef	PAVRERMRRAEPAADGV
HIV-226	Nef	RRAEPAADGVGAVSRDL
HIV-227	Nef	DGVGAVSRDLEKHGAI
HIV-228	Nef	SRDLEKHGAITSSNTAA
HIV-229	Nef	GAITSSNTAANNADCAWL
HIV-230	Nef	AANNADCAWLEAQEEEEV
HIV-231	Nef	WLEAQEEEEVGFPVRPQV
HIV-232	Nef	EVGFPVRPQVPLRPMTYK
HIV-233	Nef	QVPLRPMTYKAAVDLSHF
HIV-234	Nef	YKAAVDLSHFLKEKGGL
HIV-235	Nef	SHFLKEKGGLEGLIYSQK
HIV-236	Nef	GLEGLIYSQKRQDILDLW
HIV-237	Nef	QKRQDILDLWVYHTQGYF
HIV-238	Nef	LWVYHTQGYFPDWQNY
HIV-239	Nef	QGYFPDWQNYTPGPGIRY
HIV-240	Nef	NYTPGPGIRYPLTFGWCF
HIV-241	Nef	RYPLTFGWCFKLVPV
HIV-242	Nef	FGWCFKLVPVEPEKVEEA
HIV-243	Nef	PVEPEKVEEANEGENNSL
HIV-244	Nef	EANEGENNSLLHPMSLH
HIV-245	Nef	NSLLHPMSLHGMDDPEK
HIV-246	Nef	SLHGMDDPEKEVLVWKF
HIV-247	Nef	PEKEVLVWKFDSRLAFHH
HIV-248	Nef	KFDSRLAFHHMARELH
HIV-249	Nef	AFHHMARELHPEYYKDC
HIV-250	Rev	MAGRSGDSDEELLKTVRL
HIV-251	Rev	DEELLKTVRLIKFLY
HIV-252	Rev	KTVRLIKFLYQSNPPPS
HIV-253	Rev	FLYQSNPPPSPEGTRQAR
HIV-254	Rev	PSPEGTRQARRNRRRRW
HIV-255	Rev	QARRNRRRRWRERQRQIR
HIV-256	Rev	RWRERQRQIRSISGWIL
HIV-257	Rev	QIRSISGWILSTYLGRPA
HIV-258	Rev	ILSTYLGRPAEPVPLQL
HIV-259	Rev	RPAEPVPLQLPPLERL
HIV-260	Rev	PLQLPPLERLTLDCNED
HIV-261	Rev	ERLTLDCNEDCGTSGTQGV

HIV-262	Rev	DCGTSGTQGVGSPQIL
HIV-263	Rev	TQGVGSPQILVESPAVL
HIV-264	Rev	QILVESPAVLESGTKEE
HIV-265	Tat	MEPVDPRLEPWKHPGSQPK
HIV-266	Tat	PWKHPGSQPKTACTNCY
HIV-267	Tat	QPKTACTNCYCKKCCFH
HIV-268	Tat	NCYCKKCCFHCQVCFTTK
HIV-269	Tat	FHCQVCFTTKGLGISYGR
HIV-270	Tat	TKGLGISYGRKKRRQRRR
HIV-271	Tat	GRKKRRQRRRAPQDSQTH
HIV-272	Tat	RRAPQDSQTHQVSLSK
HIV-273	Tat	SQTHQVSLSKQPASQPR
HIV-274	Tat	LSKQPASQPRGDPTGPK
HIV-275	Tat	QPRGDPTGPKESKKKVER
HIV-276	Tat	PKESKKKVERETETDPVDQ
HIV-277	Vif	MQSLQILAIVAL
HIV-278	Vpu	LQILAIVALVVA
HIV-279	Vpu	LAIVALVVAAII
HIV-280	Vpu	IVALVVAAIIAIV
HIV-281	Vpu	IIAIVVWSIVFIEYRKIL
HIV-282	Vpu	IVFIEYRKILRQRKIDRL
HIV-283	Vpu	ILRQRKIDRLIDRIRERA
HIV-284	Vpu	RLIDRIRERAEDSGNESE
HIV-285	Vpu	RAEDSGNESEGDQEELSA
HIV-286	Vpu	SEGDQEELSALVEMGHHA
HIV-287	Vpu	SALVEMGHHAPWDVDDL
HIV-288	Pol	FFREDLAFPQGKAREF
HIV-289	Pol	AFPGKAREFSSEQTRA
HIV-290	Pol	REFSSEQTRANSPTRREL
HIV-291	Pol	RANSPTRRELQVWGR
HIV-292	Pol	TRRELQVWGRDNNSLSEA
HIV-293	Pol	GRDNNSLSEAGADRGGTV
HIV-294	Pol	EAGADRGGTVSFSFPQI
HIV-295	Pol	GTVSFSFPQITLWQRPLV
HIV-296	Pol	QITLWQRPLVTIKIGGQL
HIV-297	Pol	LVTIKIGGQLKEALL
HIV-298	Pol	IGGQLKEALLDTGADDTV
HIV-299	Pol	LLDTGADDTVLEEMNL
HIV-300	Pol	DDTVLEEMNLPGRWKPKM
HIV-301	Pol	NLPGRWKPKMIGGIGGFI
HIV-302	Pol	KMIGGIGGFIKVRQYDQI
HIV-303	Pol	FIKVRQYDQILIEICGHK
HIV-304	Pol	QILIEICGHKAIGTVLV
HIV-305	Pol	GHKAIGTVLVGPTPVNII

HIV-306	Pol	LVGPTPVNIIGRNLLTQI
HIV-307	Pol	IIGRNLLTQIGCTLNFPI
HIV-308	Pol	QIGCTLNFPISPIETVPV
HIV-309	Pol	PISPIETVPVKLKPGM
HIV-310	Pol	TVPVKLKPGMDGPKVKQW
HIV-311	Pol	GMDGPKVKQWPLTEEKIK
HIV-312	Pol	QWPLTEEKIKALVEI
HIV-313	Pol	EEKIKALVEICTEMEK
HIV-314	Pol	LVEICTEMEKEGKISKI
HIV-315	Pol	MEKEGKISKIGPENPY
HIV-316	Pol	ISKIGPENPYNTPVFAIK
HIV-317	Pol	PYNTPVFAIKKKDSTKWR
HIV-318	Pol	IKKKDSTKWRKLVDFREL
HIV-319	Pol	WRKLVDFRELNKRTQDFW
HIV-320	Pol	ELNKRTQDFWEVQLGIPH
HIV-321	Pol	FWEVQLGIPHPAGLKKKK
HIV-322	Pol	PHPAGLKKKKSVTVLDV
HIV-323	Pol	KKKSVTVLDVGDAYFSV
HIV-324	Pol	LDVGDAYFSVPLDKDFRK
HIV-325	Pol	SVPLDKDFRKYTAFTI
HIV-326	Pol	DFRKYTAFTIPSINNETPGI
HIV-327	Pol	PSINNETPGIRYQYNVL
HIV-328	Pol	PGIRYQYNVLPQGWK
HIV-329	Pol	QYNVLPQGWKGSPAIF
HIV-330	Pol	QGWKGSPAIFQSSMTKIL
HIV-331	Pol	IFQSSMTKILEPFRK
HIV-332	Pol	MTKILEPFRKQNPDIVIY
HIV-333	Pol	RKQNPDIVIYQYMDDLYV
HIV-334	Pol	IYQYMDDLYVGSDLEI
HIV-335	Pol	DLYVGSDLEIGQHRTKI
HIV-336	Pol	LEIGQHRTKIEELRQHLL
HIV-337	Pol	KIEELRQHLLRWGFTTPDK
HIV-338	Pol	LRWGFTTPDKKHQKEPPF
HIV-339	Pol	DKKHQKEPPFLWMGYELH
HIV-340	Pol	PFLWMGYELHPDKWTV
HIV-341	Pol	YELHPDKWTVQPIVLPEK
HIV-342	Pol	TVQPIVLPEKDSWTVNDI
HIV-343	Pol	EKDSWTVNDIQKLVGKL
HIV-344	Pol	NDIQKLVGKLNWASQIYA
HIV-345	Pol	KLNWASQIYAGIKVKQL
HIV-346	Pol	IYAGIKVKQLCKLLRGTK
HIV-347	Pol	QLCKLLRGTKALTEVIPL
HIV-348	Pol	TKALTEVIPLTEEAELEL
HIV-349	Pol	PLTEEAELELAENREILK

HIV-350	Pol	ELAENREILKEPVHGVYY
HIV-351	Pol	LKEPVHGVYYDPSKDLIA
HIV-352	Pol	YYDPSKDLIAEIQKQGQGQW
HIV-353	Pol	EIQKQGQGQWTYQIY
HIV-354	Pol	GQGQWTYQIYQEPFKNLK
HIV-355	Pol	IYQEPFKNLKTGKYARMR
HIV-356	Pol	LKTGKYARMRGAHTNDVK
HIV-357	Pol	MRGAHTNDVKQLTEAVQK
HIV-358	Pol	VKQLTEAVQKIATESIVI
HIV-359	Pol	QKIATESIVIWGKTPKFK
HIV-360	Pol	VIWGKTPKFKLPIQKETW
HIV-361	Pol	FKLPIQKETWEAWWTEYW
HIV-362	Pol	TWEAWWTEYWQATWIPEW
HIV-363	Pol	YWQATWIPEWEFVNRPPL
HIV-364	Pol	EWEFVNRPPLVKLWYQL
HIV-365	Pol	PPLVKLWYQLEKEPIVGA
HIV-366	Pol	QLEKEPIVGAETFYVDGA
HIV-367	Pol	GAETFYVDGAANRETKL
HIV-368	Pol	DGAANRETKLGKAGYV
HIV-369	Pol	ETKLGKAGYVTDRGRQKV
HIV-370	Pol	YVTDRGRQKVVSLTDTTNQK
HIV-371	Pol	VSLTDTTNQKTELQAIHL
HIV-372	Pol	QKTELQAIHLALQDSGL
HIV-373	Pol	IHLALQDSGLEVNIV
HIV-374	Pol	QDSGLEVNIVTDSQYAL
HIV-375	Pol	NIVTDSQYALGIIQA
HIV-376	Pol	SQYALGIIQAQPDKSESEL
HIV-377	Pol	AQPDKSESELVSQIIEQL
HIV-378	Pol	ELVSQIIEQLIKKEKVYL
HIV-379	Pol	QLIKKEKVYLAWVPAHK
HIV-380	Pol	VYLAWVPAHKGIGGNEQV
HIV-381	Pol	HKGIGGNEQVDKLVSAGI
HIV-382	Pol	QVDKLVSAGIRKVLFL
HIV-383	Pol	SAGIRKVLFLDGIDKA
HIV-384	Pol	VLFLDGIDKAQEEHEKYH
HIV-385	Pol	KAQEEHEKYHSNWRAMA
HIV-386	Pol	KYHSNWRAMASDFNLPPV
HIV-387	Pol	MASDFNLPPVVAKEIVA
HIV-388	Pol	PPVVAKEIVASCDKCQLK
HIV-389	Pol	VASCDKCQLKGEAMHGQV
HIV-390	Pol	LKGEAMHGQVDCSPGIW
HIV-391	Pol	GQVDCSPGIWQLDCTHL
HIV-392	Pol	GIWQLDCTHLEGKIILVA
HIV-393	Pol	HLEGKIILVAVHVASGYI

HIV-394	Pol	VAVHVASGYIEAEVIPA
HIV-395	Pol	GYIEAEVIPAETGQETAY
HIV-396	Pol	PAETGQETAYFLLKLAGR
HIV-397	Pol	AYFLLKLAGRWPVKTIH
HIV-398	Pol	AGRWPVKTIHTDNGSNF
HIV-399	Pol	TIHTDNGSNFTSTTVKAA
HIV-400	Pol	NFTSTTVKAACWWAGIK
HIV-401	Pol	KAACWWAGIKQEFGIPY
HIV-402	Pol	GIKQEFGIPYNPQSQGVV
HIV-403	Pol	PYNPQSQGVVESMNKELK
HIV-404	Pol	VVESMNKELKKIIGQVR
HIV-405	Pol	ELKKIIGQVRDQAEHLK
HIV-406	Pol	QVRDQAEHLKTAVQMAVF
HIV-407	Pol	LKTAVQMAVFIHNFKRK
HIV-408	Pol	AVFIHNFKRKGGIGGYSA
HIV-409	Pol	RKGGIGGYSAGERIVDII
HIV-410	Pol	SAGERIVDIIATDIQTK
HIV-411	Pol	DIIATDIQTKELQKQITK
HIV-412	Pol	TKELQKQITKIQNFRVYY
HIV-413	Pol	TKIQNFRVYYRDSRDPLW
HIV-414	Pol	YYRDSRDPLWKGPAKLLW
HIV-415	Pol	LWKGPAKLLWKGEGAVVI
HIV-416	Pol	LWKGEGAVVIQDNSDIKV
HIV-417	Pol	VIQDNSDIKVVPRRKAKI
HIV-418	Pol	KVVPRRKAKIIRDYGKQM
HIV-419	Pol	KIIRDYGKQMAGDDCVA
HIV-420	Pol	KQMAGDDCVASRQDED
HIV-421	Vpr	MEQAPEDQGPQREPYNEW
HIV-422	Vpr	GPQREPYNEWTLELLEEL
HIV-423	Vpr	EWTLELLEELKNEAVRHF
HIV-424	Vpr	ELKNEAVRHFPRIWLHSL
HIV-425	Vpr	HFPRIWLHSLGQHIYETY
HIV-426	Vpr	SLGQHIYETYGDTWAGV
HIV-427	Vpr	ETYGDTWAGVEAIIRIL
HIV-428	Vpr	AGVEAIIRILQQLLFIHF
HIV-429	Vpr	ILQQLLFIHFRIGCQHSR
HIV-430	Vpr	HFRIGCQHSRIGITQQRR
HIV-431	Vpr	SRIGITQQRRARNGASRS
HIV-432	gp120	MRVKGIRKNYQHLWRW
HIV-433	gp120	RKNYQHLWRWGTMLLGML
HIV-434	gp120	RWGTMLLGMLMICSAA
HIV-435	gp120	LGMLMICSAAEQLWVTVY
HIV-436	gp120	AAEQLWVTVYYGVPVWK
HIV-437	gp120	TVYYGVPVWKEATTTLF

HIV-438	gp120	VWKEATTTLFCASDAKAY
HIV-439	gp120	LFCASDAKAYDTEVHNVW
HIV-440	gp120	AYDTEVHNVWATHACV
HIV-441	gp120	HNVWATHACVPTDPNPQEV
HIV-442	gp120	VPTDPNPQEVVLENV
HIV-443	gp120	NVTENFNMWKNNMVEQMH
HIV-444	gp120	WKNNMVEQMHEDIISLW
HIV-445	gp120	QMHEDIISLWDQSLKPCV
HIV-446	gp120	LWDQSLKPCVKLTPLCV
HIV-447	gp120	PCVKLTPLCVTLNCTDL
HIV-448	gp120	LCVTLNCTDLNNNTNTTS
HIV-449	gp120	DLNNNTNTTSSSGEKMEK
HIV-450	gp120	TSSSGEKMEKGEIKNCSF
HIV-451	gp120	EKGEIKNCSFNITTSIR
HIV-452	gp120	CSFNITTSIRDKVQKEYA
HIV-453	gp120	IRDKVQKEYALFYKLDVV
HIV-454	gp120	YALFYKLDVVPIDNDNTSY
HIV-455	gp120	VPIDNDNTSYRLISCNTSV
HIV-456	gp120	YRLISCNTSVITQACPKV
HIV-457	gp120	SVITQACPKVSFEPIPIH
HIV-458	gp120	KVSFEPIPIHYCAPAGFA
HIV-459	gp120	IHYCAPAGFAILKCNDKK
HIV-460	gp120	FAILKCNDKKFNGTGPCTNV
HIV-461	gp120	FNGTGPCTNVSTVQCTH
HIV-462	gp120	TNVSTVQCTHGIRPVV
HIV-463	gp120	QCTHGIRPVVSTQLLL
HIV-464	gp120	RPVVSTQLLLNGSLA
HIV-465	gp120	TQLLLNGSLAEEEVVIR
HIV-466	gp120	SLAEEEVVIRSENFTDNA
HIV-467	gp120	IRSENFTDNAKTIIVQL
HIV-468	gp120	DNAKTIIVQLNESVEI
HIV-469	gp120	IVQLNESVEINCTRPNNNTR
HIV-470	gp120	NCTRPNNNTRKSIHI
HIV-471	gp120	NNNTRKSIHIGPGRAFY
HIV-472	gp120	IHIGPGRAFYTTGEII
HIV-473	gp120	RAFYTTGEIIGDIRQAH
HIV-474	gp120	EIIGDIRQAHCNISRAKW
HIV-475	gp120	AHCNISRAKWNNTLKQIV
HIV-476	gp120	KWNNTLKQIVIKLREQF
HIV-477	gp120	QIVIKLREQFGNKTIVF
HIV-478	gp120	EQFGNKTIVFNQSSGGDPEI
HIV-479	gp120	NQSSGGDPEIVMHSF
HIV-480	gp120	GDPEIVMHSFNCGGEFFY
HIV-481	gp120	SFNCGGEFFYCNTTQLF

HIV-482	gp120	FFYCNTTQLFNSTWNSTN
HIV-483	gp120	LFNSTWNSTNTEGSNNTDTI
HIV-484	gp120	TEGSNNTDTITLPCRIK
HIV-485	gp120	DTITLPCRIKQIINMW
HIV-486	gp120	CRIKQIINMWQEVGKAMY
HIV-487	gp120	MWQEVGKAMYAPPIRGQI
HIV-488	gp120	MYAPPIRGQIRCSSNI
HIV-489	gp120	RGQIRCSSNITGLLLTR
HIV-490	gp120	SNITGLLLTRDGGNNNNT
HIV-491	gp120	TRDGGNNNNTTEIFR
HIV-492	gp120	NNNNTTEIFRPGGGDMR
HIV-493	gp120	IFRPGGGDMRDNWRSELY
HIV-494	gp120	MRDNWRSELYKYKVVKI
HIV-495	gp120	ELYKYKVVKIEPLGVA
HIV-496	gp120	VVKIEPLGVAPTKAKRRV
HIV-497	gp120	VAPTKAKRRVVQREKRAV
HIV-498	gp160	RVVQREKRAVGIGAMFL
HIV-499	gp160	RAVGIGAMFLGFLGAA
HIV-500	gp160	AMFLGFLGAAGSTMGAA
HIV-501	gp160	GAAGSTMGAASMTLTVQA
HIV-502	gp160	AASMTLTVQARQLLSGIV
HIV-503	gp160	QARQLLSGIVQQQNNLLR
HIV-504	gp160	IVQQQNNLLRAIEAQQHL
HIV-505	gp160	LRAIEAQQHLQLTVWGI
HIV-506	gp160	HLQLTVWGIKQLQARVL
HIV-507	gp160	GIKQLQARVLAVERYLK
HIV-508	gp160	RVLAVERYLKDQQLLGIW
HIV-509	gp160	LKDQQLLGIWGCSGKLI
HIV-510	gp160	GIWGCSGKLICTTAVPW
HIV-511	gp160	KLICTTAVPWNASWSNK
HIV-512	gp160	VPWNASWSNKSLDQIW
HIV-513	gp160	WSNKSLDQIWNNMTWMEW
HIV-514	gp160	IWNNMTWMEWEREIDNY
HIV-515	gp160	MEWEREIDNYTSLIYTLI
HIV-516	gp160	NYTSLIYTLIEESQNQQEK
HIV-517	gp160	IEESQNQQEKNEQELLEL
HIV-518	gp160	EKNEQELLELDKWASLW
HIV-519	gp160	LELDKWASLWNWFDITNW
HIV-520	gp160	LWNWFDITNWLWYIKIFI
HIV-521	gp160	NWLWYIKIFIMIVGGLV
HIV-522	gp160	IFIMIVGGLVGLRIVFAV
HIV-523	gp160	LVGLRIVFAVLSIVNRVR
HIV-524	gp160	AVLSIVNRVRQGYSPLSF
HIV-525	gp160	VRQGYSPLSFQTRLPAPR

HIV-526	gp160	SFQTRLPAPRGPDRPEGI
HIV-527	gp160	PRGPDRPEGIEEEGGER
HIV-528	gp160	EGIEEEGGERDRDRSQRL
HIV-529	gp160	ERDRDRSQRLVDGFLALI
HIV-530	gp160	RLVDGFLALIWVDLRSL
HIV-531	gp160	ALIWVDLRSLCLFSYHRL
HIV-532	gp160	SLCLFSYHRLRDLLLIV
HIV-533	gp160	HRLRDLLLIVTRIVELL
HIV-534	gp160	LIVTRIVELLGRRGWEAL
HIV-535	gp160	LLGRRGWEALKYWWNLL
HIV-536	gp160	EALKYWWNLLQYWSQELK
HIV-537	gp160	LLQYWSQELKNSAVSLL
HIV-538	gp160	ELKNSAVSLLNATAIAVA
HIV-539	gp160	LLNATAIAVAEGTDRVI
HIV-540	gp160	AVAEGTDRVIEVVQRACR
HIV-541	gp160	VIEVVQRACRAILHIPRR
HIV-542	gp160	CRAILHIPRRIRQGL
HIV-543	gp160	HIPRRIRQGLERALL
HIV-544	Vif	MENRWQVMIVWQVDRMRI
HIV-545	Vif	IVWQVDRMRIRTWKSLVK
HIV-546	Vif	RIRTWKSLVKHHMYISGK
HIV-547	Vif	VKHHMYISGKAKGWFYRH
HIV-548	Vif	GKAKGWFYRHHYESTHPR
HIV-549	Vif	RHHYESTHPRISSEVHI
HIV-550	Vif	HPRISSEVHIPLGDARLV
HIV-551	Vif	HIPLGDARLVITTYWGL
HIV-552	Vif	RLVITTYWGLHTGERDWHL
HIV-553	Vif	LHTGERDWHLGQGVSIEW
HIV-554	Vif	HLGQGVSIEWRKKRY
HIV-555	Vif	VSIEWRKKRYSTQVDPDL
HIV-556	Vif	LIHLYYFDCFSESAIRNA
HIV-557	Vif	CFSESAIRNAILGHIV
HIV-558	Vif	IRNAILGHIVSPRCEYQA
HIV-559	Vif	IVSPRCEYQAGHNKVGSL
HIV-560	Vif	SLQYLALAALITPKKIK
HIV-561	Vif	IKPPLPSVTKLTEDRWNK
HIV-562	Vif	TKLTEDRWNKPQKTKGHR
HIV-563	Vif	NKPQKTKGHRGSHTMNGH

Table 3. Library of synthetic peptides described to bind to HLA-C*06:02

Name	Type	Sequence
HLACW6-FL9	synthetic	FRPDLVSML
HLACW6-FD9	synthetic	FRAEDNLLG
HLACW6-YT9	synthetic	YRPDTPHQI
HLACW6-GV9	synthetic	GRMMVKIQA
HLACW6-SV9	synthetic	SRGPVHHLL
HLACW6-FI9	synthetic	FRDAINSFG
HLACW6-YH9	synthetic	YRFNHGTLF
HLACW6-FQ9	synthetic	FRAWQAALV
HLACW6-FI9	synthetic	FQYWIERDF
HLACW6-FE9	synthetic	FRSAEIKAL
HLACW6-MF9	synthetic	MTKPFTVDL
HLACW6-FG9	synthetic	FRMAGFMNV
HLACW6-YG9	synthetic	YRMNGSSQI
HLACW6-YA9	synthetic	YRNQALIAI
HLACW6-FR9	synthetic	FGMERNYLF
HLACW6-FQ9	synthetic	FRAEQAYNV
HLACW6-FS9	synthetic	FRNISIRWI
HLACW6-FH9	synthetic	FYRWHPGEY
HLACW6-YG9	synthetic	YRYHGVQG

Table 4. SIV peptides library (SIV macaque sequence, isolate 239).

Name	Type	Sequence
SIVmac 239 -1	GAG	MGVRNSVLSGKKADE
SIVmac 239 -2	GAG	NSVLSGKKADELEKI
SIVmac 239 -3	GAG	SGKKADELEKIRLRP
SIVmac 239 -4	GAG	ADELEKIRLRPNGKK
SIVmac 239 -5	GAG	EKIRLRPNGKKKYML
SIVmac 239 -6	GAG	LRPNGKKKYMLKHVV
SIVmac 239 -7	GAG	GKKKYMLKHVVWAAN
SIVmac 239 -8	GAG	YMLKHVVWAANELDR
SIVmac 239 -9	GAG	HVVWAANELDRFGLA
SIVmac 239 -10	GAG	AANELDRFGLAESLL
SIVmac 239 -11	GAG	LDRFGLAESLLENKE
SIVmac 239 -12	GAG	GLAESLLENKEGCQK
SIVmac 239 -13	GAG	SLLENKEGCQKILSV
SIVmac 239 -14	GAG	NKEGCQKILSVLAPL
SIVmac 239 -15	GAG	CQKILSVLAPLVPTG
SIVmac 239 -16	GAG	LSVLAPLVPTGSENL
SIVmac 239 -17	GAG	APLVPTGSENLKSLY
SIVmac 239 -18	GAG	PTGSENLKSLYNTVC
SIVmac 239 -19	GAG	ENLKSLYNTVCVIWC
SIVmac 239 -20	GAG	SLYNTVCVIWCIHAE
SIVmac 239 -21	GAG	TVCVIWCIHAEEKVK
SIVmac 239 -22	GAG	IWCIHAEEKVKHTEE
SIVmac 239 -23	GAG	HAEEKVKHTEEAKQI
SIVmac 239 -24	GAG	KVKHTEEAKQIVQRH
SIVmac 239 -25	GAG	TEEAKQIVQRHLVVE
SIVmac 239 -26	GAG	KQIVQRHLVVETGTT
SIVmac 239 -27	GAG	QRHLVVETGTTETMP
SIVmac 239 -28	GAG	VVETGTTETMPKTSR
SIVmac 239 -29	GAG	GTTETMPKTSRPTAP
SIVmac 239 -30	GAG	TMPKTSRPTAPSSGR
SIVmac 239 -31	GAG	TSRPTAPSSGRGGNY
SIVmac 239 -32	GAG	TAPSSGRGGNYPVQQ
SIVmac 239 -33	GAG	SGRGGNYPVQQIGGN
SIVmac 239 -34	GAG	GNYPVQQIGGNYVHL
SIVmac 239 -35	GAG	VQQIGGNYVHLPLSP
SIVmac 239 -36	GAG	GGNYVHLPLSPRTLN
SIVmac 239 -37	GAG	VHLPLSPRTLNAWVK
SIVmac 239 -38	GAG	LSPRTLNAWVKLIEE
SIVmac 239 -39	GAG	TLNAWVKLIEEKKFG
SIVmac 239 -40	GAG	WVKLIEEKKFGAEVV

SIVmac 239 -41	GAG	IEEKKFGAEVVPGFQ
SIVmac 239 -42	GAG	KFGAEVVPGFQALSE
SIVmac 239 -43	GAG	EVVPGFQALSEGCTP
SIVmac 239 -44	GAG	GFQALSEGCTPYDIN
SIVmac 239 -45	GAG	LSEGCTPYDINQMLN
SIVmac 239 -46	GAG	CTPYDINQMLNCVGD
SIVmac 239 -47	GAG	DINQMLNCVGDHQAA
SIVmac 239 -48	GAG	MLNCVGDHQAAMQII
SIVmac 239 -49	GAG	VGDHQAAMQIIRDII
SIVmac 239 -50	GAG	QAAMQIIRDIINEEA
SIVmac 239 -51	GAG	QIIRDIINEEAADWD
SIVmac 239 -52	GAG	DIINEEAADWDLQHP
SIVmac 239 -53	GAG	EEAADWDLQHPQPAP
SIVmac 239 -54	GAG	DWDLQHPQPAPQQGQ
SIVmac 239 -55	GAG	QHPQPAPQQGQLREP
SIVmac 239 -56	GAG	PAPQQGQLREPSGSD
SIVmac 239 -57	GAG	QGQLREPSGSDIAGT
SIVmac 239 -58	GAG	REPSGSDIAGTTSSV
SIVmac 239 -59	GAG	GSDIAGTTSSVDEQI
SIVmac 239 -60	GAG	AGTTSSVDEQIQWMY
SIVmac 239 -61	GAG	SSVDEQIQWMYRQQN
SIVmac 239 -62	GAG	EQIQ WMYRQQNPIPV
SIVmac 239 -63	GAG	WMYRQQNPIPVGNIY
SIVmac 239 -64	GAG	QQNPIPVGNIYRRWI
SIVmac 239 -65	GAG	IPVGNIYRRWIQLGL
SIVmac 239 -66	GAG	NIYRRWIQLGLQKCV
SIVmac 239 -67	GAG	RWIQLGLQKCVRMYN
SIVmac 239 -68	GAG	LGLQKCVRMYNPTNI
SIVmac 239 -69	GAG	KCVRMYNPTNILDVK
SIVmac 239 -70	GAG	MYNPTNILDVKQGPK
SIVmac 239 -71	GAG	TNILDVKQGPKEPFQ
SIVmac 239 -72	GAG	DVKQGPKEPFQSYVD
SIVmac 239 -73	GAG	GPKEPFQSYVDRFYK
SIVmac 239 -74	GAG	PFQSYVDRFYKSLRA
SIVmac 239 -75	GAG	YVDRFYKSLRAEQTD
SIVmac 239 -76	GAG	FYKSLRAEQTDAAVK
SIVmac 239 -77	GAG	LRAEQTDAAVKNWMT
SIVmac 239 -78	GAG	QTDAAVKNWMTQTLL
SIVmac 239 -79	GAG	AVKNWMTQTLLIQNA
SIVmac 239 -80	GAG	WMTQTLLIQNANPDC
SIVmac 239 -81	GAG	TLLIQNANPDCKLVL
SIVmac 239 -82	GAG	QNANPDCKLVLKGLG

SIVmac 239 -83	GAG	PDCKLVLKGLGVNPT
SIVmac 239 -84	GAG	LVLKGLGVNPTLEEM
SIVmac 239 -85	GAG	GLGVNPTLEEMLTAC
SIVmac 239 -86	GAG	NPTLEEMLTACQGVG
SIVmac 239 -87	GAG	EEMLTACQGVGGPGQ
SIVmac 239 -88	GAG	TACQGVGGPGQKARL
SIVmac 239 -89	GAG	GVGGPGQKARLMAEA
SIVmac 239 -90	GAG	PGQKARLMAEALKEA
SIVmac 239 -91	GAG	ARLMAEALKEALAPV
SIVmac 239 -92	GAG	AEALKEALAPVPIPF
SIVmac 239 -93	GAG	KEALAPVPIPFAAAQ
SIVmac 239 -94	GAG	APVPIPFAAAQQRGP
SIVmac 239 -95	GAG	IPFAAAQQRGPRKPI
SIVmac 239 -96	GAG	AAQQRGPRKPIKCWN
SIVmac 239 -97	GAG	RGPRKPIKCWNCGKE
SIVmac 239 -98	GAG	KPIKCWNCGKEGHSA
SIVmac 239 -99	GAG	CWNCGKEGHSARQCR
SIVmac 239 -100	GAG	GKEGHSARQCRAPRR
SIVmac 239 -101	GAG	HSARQCRAPRRQGCW
SIVmac 239 -102	GAG	QCRAPRRQGCWKCGK
SIVmac 239 -103	GAG	PRRQGCWKCGKMDHV
SIVmac 239 -104	GAG	GCWKCGKMDHVMAKC
SIVmac 239 -105	GAG	CGKMDHVMAKCPDRQ
SIVmac 239 -106	GAG	DHVMAKCPDRQAGFL
SIVmac 239 -107	GAG	AKCPDRQAGFLGLGP
SIVmac 239 -108	GAG	DRQAGFLGLGPWGKK
SIVmac 239 -109	GAG	GFLGLGPWGKKPRNF
SIVmac 239 -110	GAG	LGPWGKKPRNFPMAQ
SIVmac 239 -111	GAG	GKKPRNFPMAQVHQG
SIVmac 239 -112	GAG	RNFPMAQVHQGLMPT
SIVmac 239 -113	GAG	MAQVHQGLMPTAPPE
SIVmac 239 -114	GAG	HQGLMPTAPPEDPAV
SIVmac 239 -115	GAG	MPTAPPEDPAVDLLK
SIVmac 239 -116	GAG	PPEDPAVDLLKNYMQ
SIVmac 239 -117	GAG	PAVDLLKNYMQLGKQ
SIVmac 239 -118	GAG	LLKNYMQLGKQQREK
SIVmac 239 -119	GAG	YMQLGKQQREKQRES
SIVmac 239 -120	GAG	GKQQREKQRESREKP
SIVmac 239 -121	GAG	REKQRESREKPYKEV
SIVmac 239 -122	GAG	RESREKPYKEVTEDL
SIVmac 239 -123	GAG	EKPYKEVTEDLLHLN
SIVmac 239 -124	GAG	KEVTEDLLHLNSLFG

SIVmac 239 -125	GAG	EDLLHLNSLFGGDQ

Table 5. EBOLA peptides library (Kikwit strain, GP protein).

Name	Type	Sequence
EB-GP-Z-3	GP	LPRDRFKRTSFFLWV
EB-GP-Z-4	GP	RFKRTSFFLWVIILF
EB-GP-Z-36	GP	PDGIRGFPRCRYVHK
EB-GP-Z-37	GP	RGFPRCRYVHKV
EB-GP-Z-38	GP	GFPRCRYVHKVSGTG
EB-GP-Z-67	GP	ETEYLFEVDNLTYV
EB-GP-Z-71	GP	VQLESRFTPQFLLQL

Table 6. hCMV peptides library (strain AD169, 65kDa phosphoprotein).

Name	Type	Sequence
hCMV-1	UL83/pp65	CSMENTRLV
hCMV-2	UL83/pp65	FQYEIMNYF
hCMV-3	UL83/pp65	FRKEVNSQL
hCMV-4	UL83/pp65	FRRRHRQDA
hCMV-5	UL83/pp65	MYMCYRRKM
hCMV-6	UL83/pp65	YAYIYTTYL
hCMV-7	UL83/pp65	YMCYRRKMM
hCMV-8	UL83/pp65	YMCYRRRKM
hCMV-9	UL83/pp65	YRHTWDRYR
hCMV-10	UL83/pp65	YRIQGKLEI
hCMV-11	UL83/pp65	YRIQGKLEY
hCMV-12	UL83/pp65	YRNMIIHAY
hCMV-13	UL83/pp65	YRRRKMMYM
hCMV-14	UL83/pp65	FRCPEMISV
hCMV-15	UL83/pp65	FRTLLVYLL
hCMV-16	UL83/pp65	SRHVRVSQP
hCMV-17	UL83/pp65	SRMVTNLMA
hCMV-18	UL83/pp65	YRIFAQEPM
hCMV-19	UL83/pp65	YRIFDANDI
hCMV-20	UL83/pp65	YRIQGKLPV
hCMV-21	UL83/pp65	YRIRETVEL