学位論文
Doctoral Thesis

Control of HIV-1 by NK cells via KIR2DL2

(KIR2DL2 を介した NK 細胞による HIV-1 の感染制御)

林　湛松
Zhansong Lin

指導教員

滝口　雅文　教授

熊本大学大学院医学教育部博士課程医学専攻エイズ学 I

2016年度

学　位　論　文

Doctoral Thesis

論文題名　：　Control of HIV-1 by NK cells via KIR2DL2
(KIR2DL2 を介した NK 細胞による HIV-1 の感染制御)

著 者 名　：　林　湛松
Zhansong　Lin

指導教員名　：　熊本大学大学院医学教育部博士課程医学専攻エイズ学Ⅰ　滝口　雅文　教授

審査委員名　：　エイズ学Ⅳ担当教授　鈴　伸也
エイズ学Ⅱ担当教授　松下　修三
エイズ学Ⅴ担当教授　上野　貴将
エイズ学ⅩⅢ担当教授　佐藤　賢文

２０１６年度

TABLE OF CONTENTS

Contents **Page**

1. ABSTRACT

[Background and purpose] Killer cell immunoglobulin-like receptors (KIRs) play an important role in the regulation of NK cell anti-viral functions and influence the clinical outcome of HIV-1 infection. In the present study, we studied the effect of KIR-HLA interaction on NK cell reactivation and eradication of HIV-1 infection in the Japanese population.

[Methods] We analyzed the correlation between the clinical outcomes of HIV-1 infection and the presence of different KIR genes or KIR-HLA combinations in a cohort consist of 504 treatment naïve HIV-1 chronically infected Japanese patients. In order to clarify the mechanisms of the protective effect of KIR genes or KIR-HLA combinations, we then performed viral suppression assay, NK cell functional assay, peptide-HLA binding assay and pHLA-KIR binding assay.

[Results] The genetic analysis identified two KIR/HLA combinations, KIR2DL2/HLA-C*12:02 and KIR2DL2/HLA-C*14:03, with synergistic effect on suppression of HIV-1 replication. Viral suppression analysis showed that KIR2DL2^{+} NK cells inhibited viral replication significantly stronger than KIR2DL2^{-} NK cells in HLA-C*14:03^{+} or HLA-C*12:02^{+} cells infected with the WT HIV-1 or the virus with an escape mutation. Binding assay suggested that the reduced peptide-HLA binding and the lower pHLA surface expression level directly influences NK cell recognition of the target cells and reactivation via KIR2DL2.

[Discussions] We showed that peptide-HLA binding and surface expression directly influence KIR2DL2^{+} NK cell reactivation which is different from previous studies on Caucasian and African cohorts suggesting that the binding affinity of inhibitory KIRs to the pHLA influences NK cell activity.

[Conclusions] We demonstrated the synergistic effect of KIR2DL2/HLA-C*12:02 and KIR2DL2/HLA-C*14:03 on HIV-1 control as well as the role of NK cells via KIR recognition in HIV-1 infection.

2. PUBLICATION LIST

I Reference Publications

Zhansong Lin, Kimiko Kuroki, Nozomi Kuse, Xiaoming Sun, Tomohiro Akahoshi, Ying Qi, Takayuki Chikata, Takuya Naruto, Madoka Koyanagi, Hayato Murakoshi, Hiroyuki Gatanaga, Shinichi Oka, Mary Carrington, Katsumi Maenaka, Masafumi Takiguchi. HIV-1 Control by NK Cells via Reduced Interaction between KIR2DL2 and HLA-C*12:02/C*14:03, *Cell Reports* 17:2210-2220, 2016

3. AKNOWLEGEMENTS

Foremost, I would like to express my sincere gratitude to my supervisor Dr. Masafumi Takiguchi (Professor, Center for AIDS Research, Kumamoto University) for the continuous support of my PhD study and research, for his patience, motivation, enthusiasm, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better supervisor for my PhD study.

Besides my supervisor, I would like extend my gratitude to my advisors: Prof. Shuzo Matsushita and Prof. Dr. Shinichi Oka, for their encouragement, insightful suggestions and questions.

I am most grateful to Dr. Mary Carrington, Cancer and Inflammation Program, Laboratory of Experimental Immunology, Leidos Biomedical Research, Inc., Frederick National Laboratories for Cancer Research, Frederick, Maryland, USA, for her valuable advices during this thesis work and this work would not be achievable without her support and guidance. I really feel great honor to have such collaborative work with her.

My sincere thanks also go to Dr. Hiroyuki Gatanaga, AIDS Clinical Center, National Center for Global Health and Medicine, Shinjuku-ku, Tokyo, Japan. He and Dr. Shinichi Oka kindly provided me priceless clinical samples for my study. I would like to thank Prof. Katsumi Maenaka, Dr. Kimiko Kuroki and Dr. Madoka Koyanagi, for their collaborative work and help in my study.

I would like to thank Dr. Keiko Sakai, Dr. Takuya Naruto, Dr. Yoshinori Sato, Dr. Tomohiro Akahoshi, Dr. Nozomi Kuse, Dr. Xiaoming Sun, Dr. Hayato Murakoshi and Dr. Takayuki Chikata for their patience and proper guidance when I started my research in a totally new environment and make me mastered in every new technique.

I would like to thank Arif, Giang, Chengcheng Zou, Yu Zhang, Trang and all laboratory members for their nice and generous help during my PhD study in Kumamoto. I would like to thank Sachiko Sakai and Sachie Kawazoe for their kind cooperation and secretarial work.

I would like to thank “MEXT” for ranting me the scholarship to study and live in Kumamoto, Japan.

Last but not the least; I would like to express my sincere gratitude to all my family members, all friends for their moral support and inspiration throughout this period of time.

4. ABBREVIATIONS

AA	Amino acid
AIDS	Acquired Immunodeficiency Syndrome
ART	Anti-retroviral Therapy
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocyte
ELISA	Enzyme-linked immunosorbent assay
HAART	Highly Active Anti-retroviral Therapy
HCV	Hepatitis C virus
HIV	Human Immunodeficiency Virus
HLA	Human leukocyte antigen
IQR	Interquartile range
KIR	Killer cell immunoglobulin-like receptor
LD	Linkage disequilibrium
LTNP	Long Term Non-Progressor
MACS	Magnetic-activated cell sorting
MHC	Major histocompatibility complex
MFI	Mean fluorescence intensity
Nef	Negative Regulatory Factor
NK	Natural killer

PBMC	Peripheral blood mononuclear cell
PE	phycoerythrin
PCR	Polymerase chain reaction
pHLA	peptide-HLA complex
pVL	plasma viral load
rIL-2	recombinant interleukin-2
RU	Relative units
SIV	Simian Immunodeficiency Virus
TCR	T cell receptor
WT	Wild type

5. BACKGROUND AND OBJECTIVES

5-1) NK cell and innate immune system

Natural killer (NK) cells are large granular lymphocytes which share the same progenitor as B cells and T cells (Fig. A); they kill tumor cells or viral-infected cells by cytokine secretion and/or by mediating cytolytic functions without any prior sensitization. Therefore, NK cells are mainly classified as a member of the innate immune system. However, unlike other components in the innate immune responses, NK cells also show a kind of unique and distinct “specificity” [1-4] and “memory” [5-7] mediated by the interaction between killer cell immunoglobulin-like receptors (KIRs) expressed on the NK cell and human leukocyte antigen class I (HLA class I) molecules expressed on the potential target cell. In sum, NK cells actually serve in the interface between the innate and the acquired immune system. In the past several decades, it has been widely reported that, NK cells play an important role in the eradication of target cells infected with different species of viruses, such as HCV [8, 9], human Hantavirus [10], Epstein-Barr virus [11], cytomegalovirus [12], influenza A virus [13], Ebola virus [14] and HIV-1.

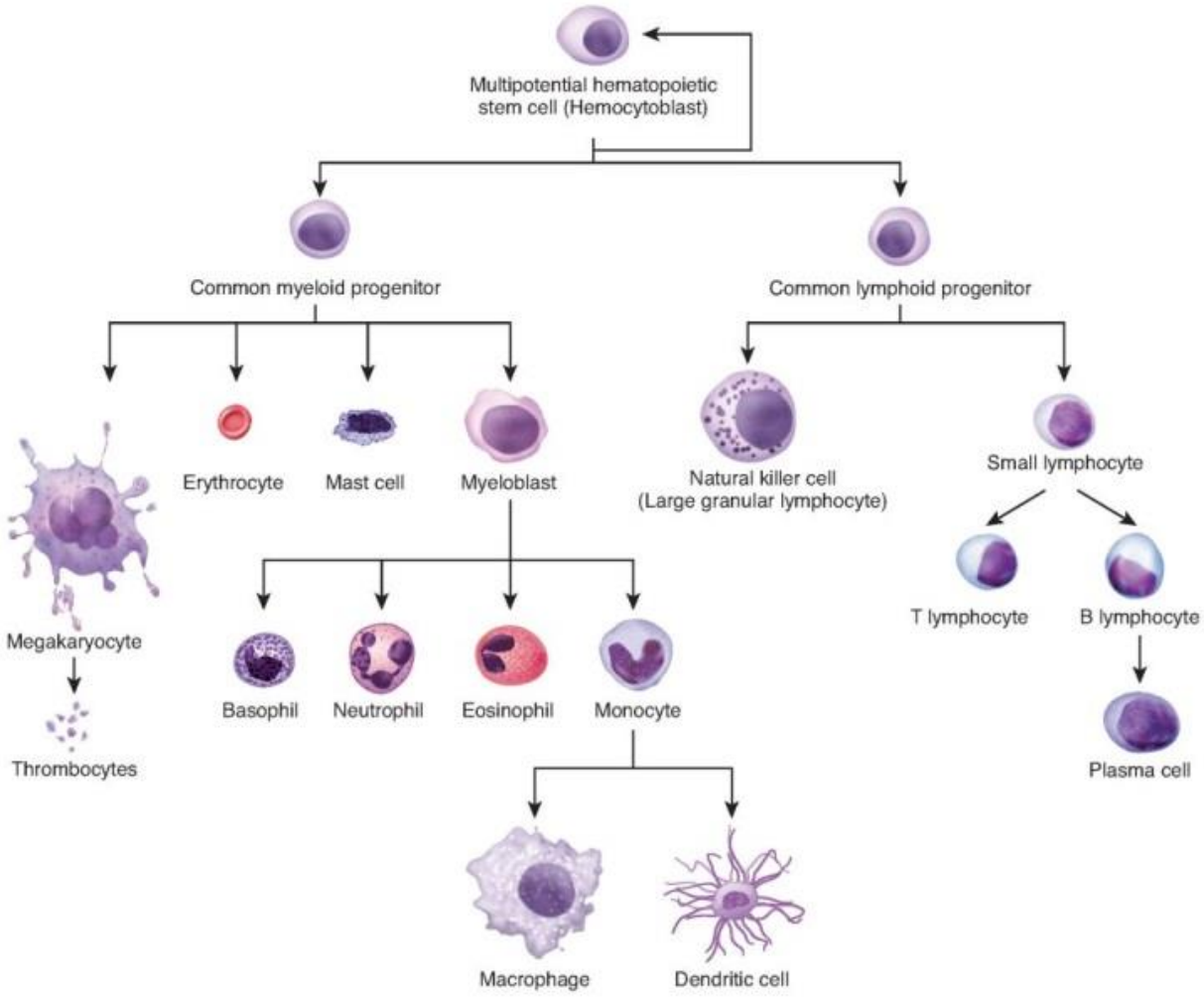


Figure A. The process of hematopoiesis. The multipotent hematopoietic stem cells give rise to many different cell types, including the cells of the immune system and red blood cells. NK cells share the same progenitor as B cells and T cells which is termed as common lymphoid progenitor. The figure is adapted from "*Anatomy and Physiology I*" by OpenStax College , http://www.bsonbooks.com/Books/B0/27/BIGF/I/0337_Hematopoiesis_new.jpg

5-2) Killer cell immunoglobulin-like receptors (KIRs)

KIR genes are encoded within a 150 Kb stretch of the 1 Mb long extended LRC (Leukocyte Receptor Complex) on chromosome 19 (19q13.4) (Fig. B). The extended LRC also contains the genes encoding some other famous receptors of human immune system, e. g., DAP adaptor proteins, CD66 antigens as well as SIGLEC, FcGRT, LILR, LAIR, FcAlphaR and NCR1

receptors. However, it is noticeable that the gene of HLA which is the ligand for KIR located on the different chromosome (chromosome 6).

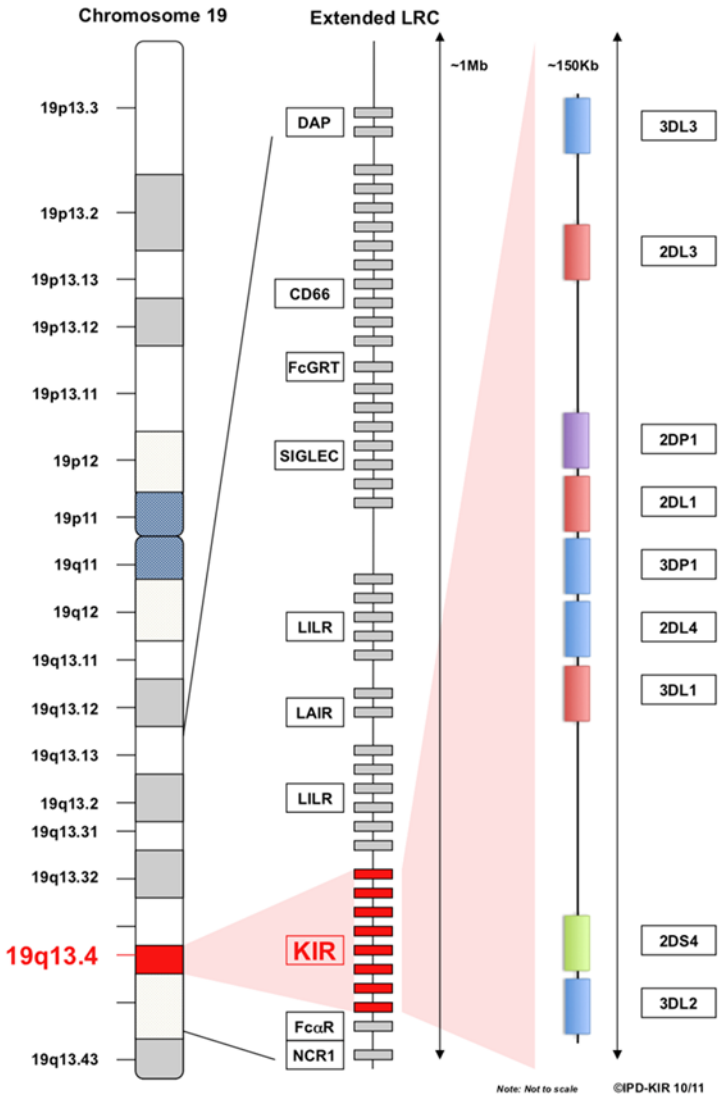


Figure B. The location of KIR gene complex on human chromosome 19q13.4. The figure is adapted from Immuno Polymorphism Database, https://www.ebi.ac.uk/ipd/kir/images/figure_01.png

Like HLA class I genes, KIR genes also show extremely high polymorphisms as reflected by the diversity and variability in both gene number and gene content [15] (Fig. C). There are totally 16 KIR genes including 2 pseudo-genes, 2DP1 and 3DP1, which do not express on the cell

surface. Based on the strong linkage disequilibrium (LD), KIR genes can be divided into two haplotypes: Haplotype A, with limited and invariable gene number and gene content and haplotype B, with diverse and variable gene number and gene content.

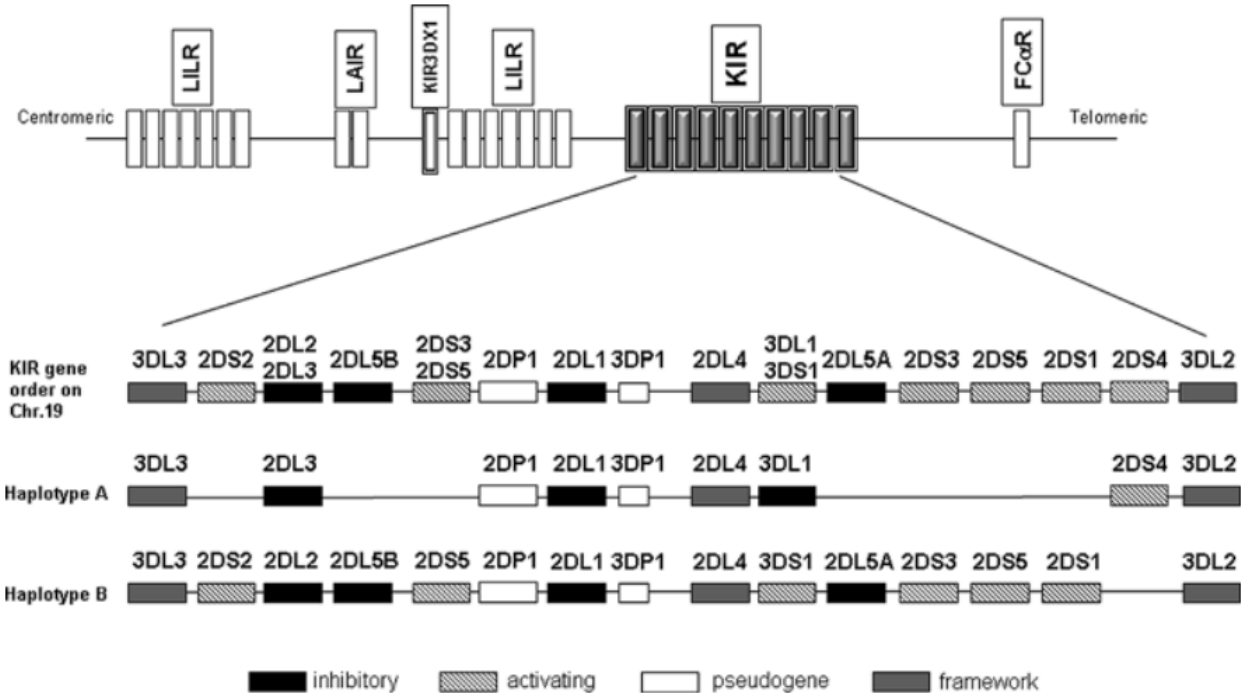


Figure C. The two haplotypes of KIR genes. The A haplotype is fixed in terms of gene content, while B haplotype is characterized by variable gene numbers. Black boxes represent inhibitory receptors, hatched boxes represent activating receptors, open boxes represent pseudogenes, and gray boxes represent framework loci present on virtually all haplotypes. The figure is adapted from Kulkarni et al., 2010 [16].

Based on the extracellular domains, KIRs can be divided into two groups: KIRs with 3 extracellular domains which recognize HLA-A/B alleles are nominated as KIR3D; whereas KIRs with only two extracellular domains which recognize HLA-C alleles are named as KIR2D (Fig. D). Based on the intracellular domain, KIRs can be divided into two families: KIRs with a shorter intracellular domain (KIR2DS/3DS) transducing stimulatory signals by an immuno-receptor tyrosine-based activating motif (ITAM) belong to the activating KIR family; whereas KIRs with a longer intracellular domain (KIR2DL/3DL) transducing inhibitory signals by an

immuno-receptor tyrosine-based inhibitory motif (ITIM) belong to the inhibitory KIR family (Fig. D).

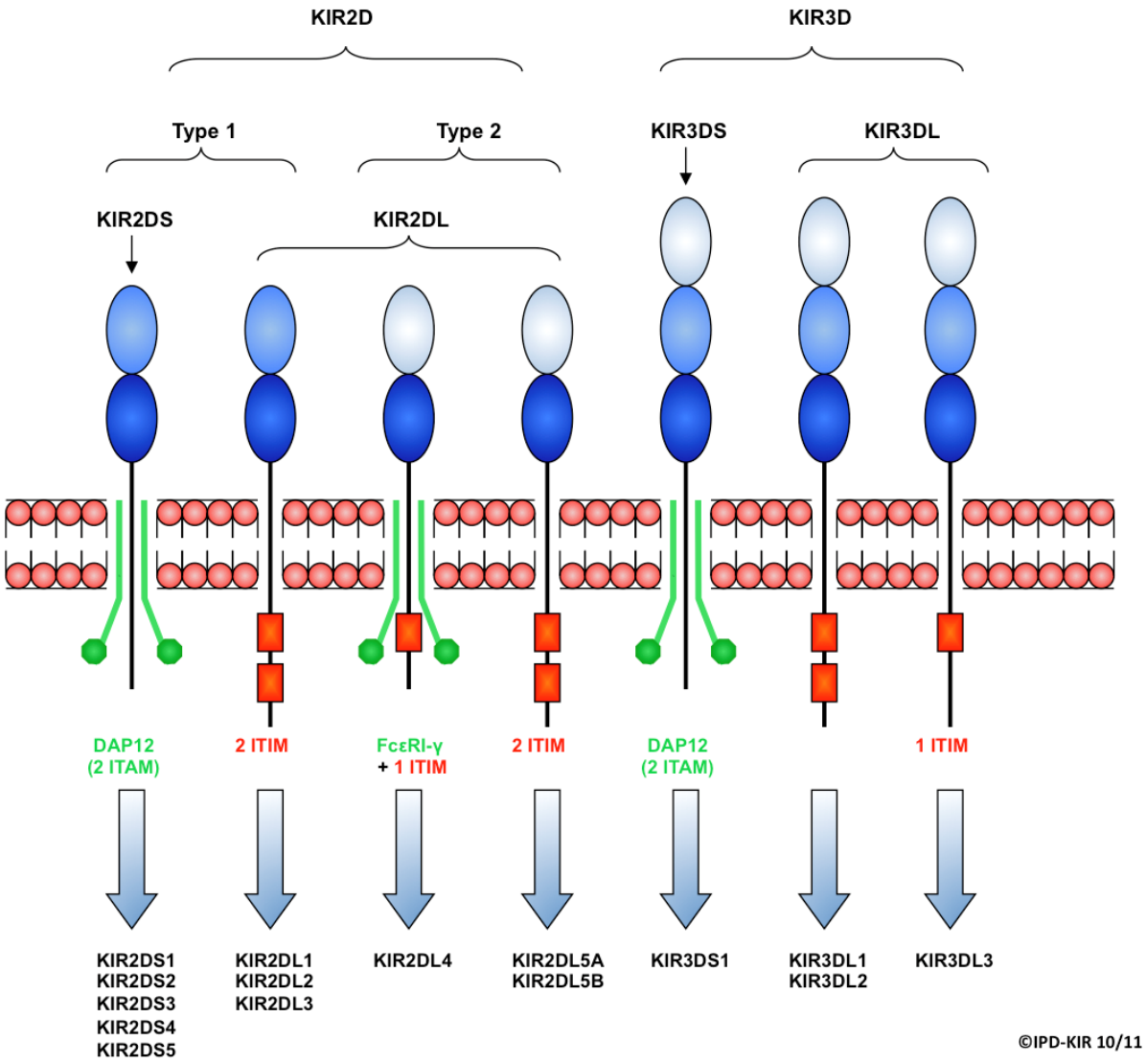


Figure D. KIR protein structures. The structural characteristics of two and three Ig-like domain KIR proteins are shown. The association of activating KIR to adaptor molecules is shown in green, whereas the ITIM of inhibitory KIR are shown as red boxes. The figure is adapted from Immuno Polymorphism Database, https://www.ebi.ac.uk/ipd/kir/images/figure_01.png

5-3) KIR-HLA interaction

The interaction between KIRs especially inhibitory KIRs and their HLA ligands play an important role in the regulation of NK cell maturation (education) and functions. In the course of NK cell functional maturation which is also termed as NK cell license or NK cell education, cells that cannot recognize self-HLA molecules by inhibitory KIRs would ultimately turn hyposensitive; only the cells that can sense self-HLA molecules gain effector functions responding effectively to the delicate and intricate balance between the stimulatory and inhibitory signals [17-23]. Also, KIR/HLA interaction directs NK cell functional activation. Under normal conditions, inhibitory KIRs expressed on the NK cells strongly "sense" the HLA ligands expressed on the potential target cells conferring the dominance of inhibitory signal; once the interaction between inhibitory KIRs and HLA ligands decreases or even vanishes, such as down-regulation of HLA molecules on the target cells resulted from HIV-1 infection, NK cells lose the strong inhibition which facilitates the activation of NK cells and leads to the subsequent lysis of stressed target cells [24-28] (Fig. E).

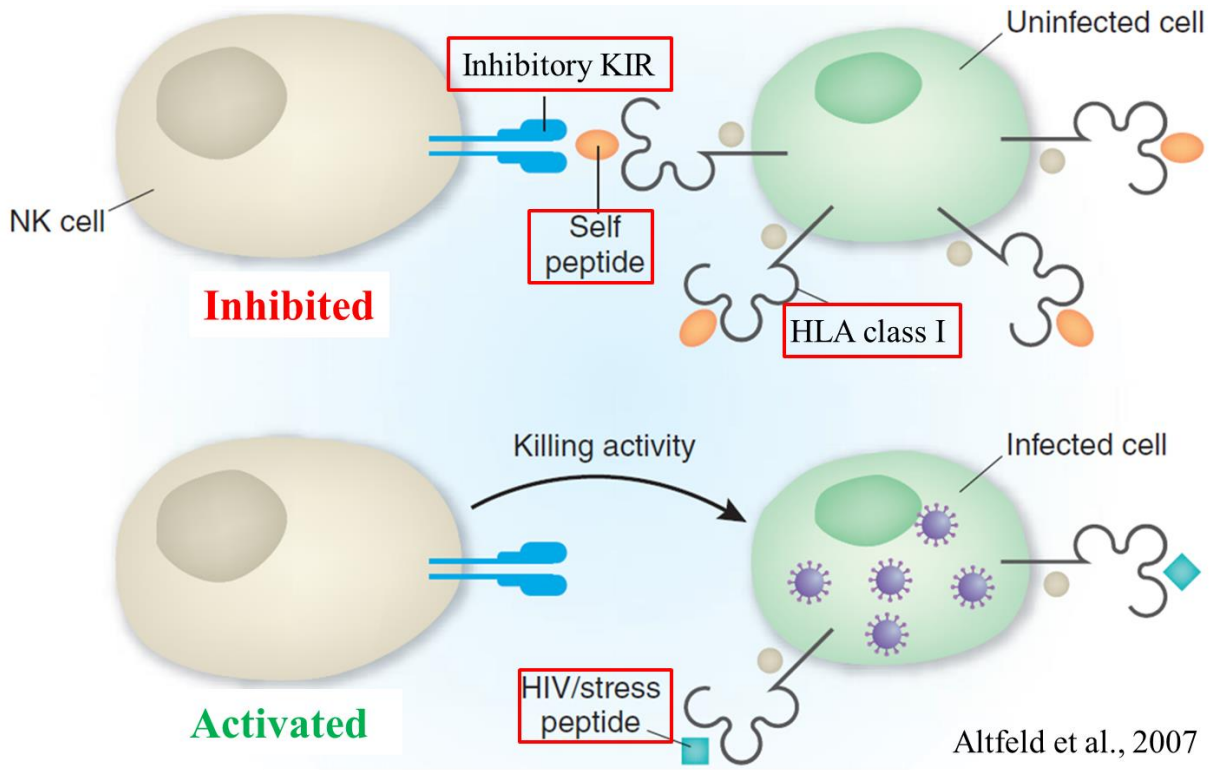


Figure E. The activation of NK cells is regulated by the interaction between KIR and pHLA complex. Under normal circumstances, inhibitory KIRs and HLA class I molecules presenting self-peptides strongly bind together and confer strong inhibition of NK cell function (the upper panel). After viral infection, inhibitory KIRs lose the binding to peptide-HLA complexes and result in the dis-inhibition (activation) of NK cells (the lower panel). The figure is adapted from Altfeld et al., 2007 [28] with slight modification.

The polymorphism in both KIR and their HLA ligands make it extremely different in the response of NK cells to pathogens between ethnic groups or even between individuals in the same ethnic group. The co-presence of KIR3DL1/S1 and its ligand Bw4-80I (HLA-A/B alleles which belong to the Bw4 group and contain an isoleucine at position 80) confer stronger killing of infected cells and the consequent better clinical outcomes or slower disease progression in HIV-1 infection [29-34] (Fig. F).

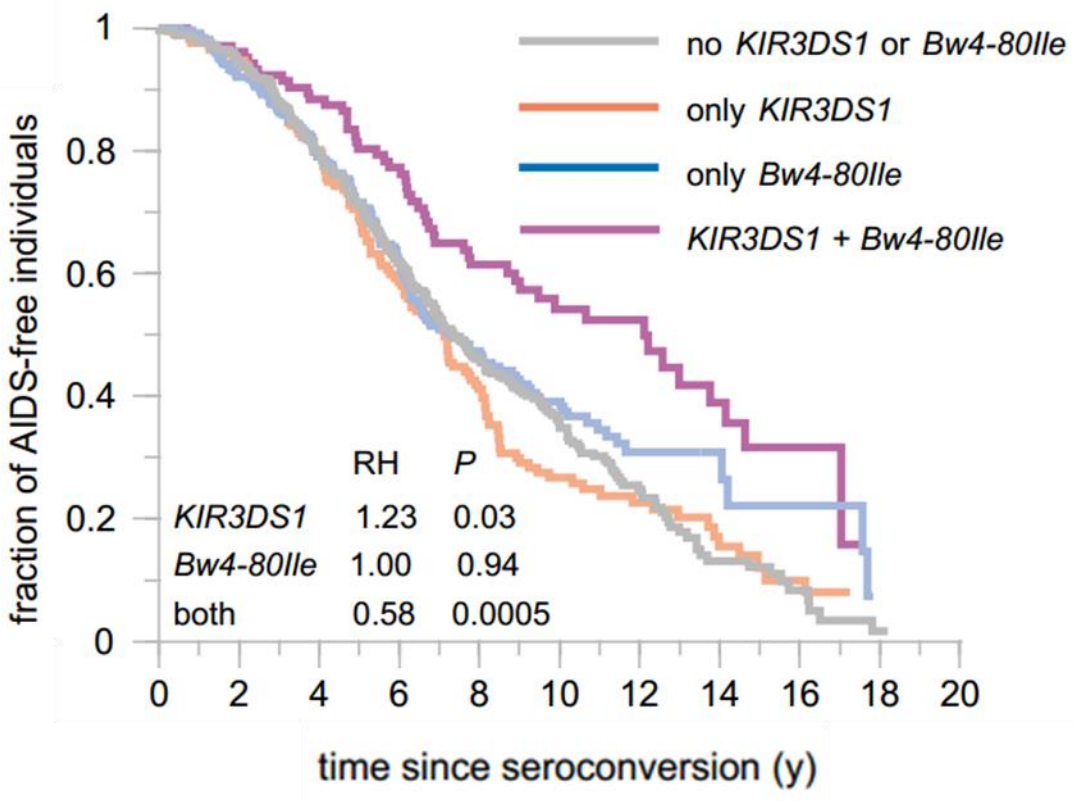


Figure F. The co-presence of KIR3DS1 and its ligand Bw4-80I results in better clinical outcome in HIV-1 infection. The longitudinal study showed that KIR3DS1/HLA-Bw4-80I confers Delayed progression to AIDS. The figure is adapted from Martin et al., *Nat Genet*, 2002 [35].

By screening a large cohort of HCV infected patients, Khakoo et al. discovered that KIR2DL3 positive individuals homozygous for HLA-C1 (HLA-C alleles with asparagine on position 80) had better clinical outcomes which suggested the synergistic protective effect of the KIR2DL3 and HLA-C1 combination on HCV infection [9].

Like HLA class I alleles, KIRs independently or combined with their HLA ligands select escape mutations in virus sequences. Alter et al. discovered that polymorphisms at two positions in the vpu region of HIV-1 sequence were significantly enriched in individuals possessing both

KIR2DL2 and HLA-C1 which suggested the immune pressure conferred by the synergistic influence of KIRs and HLA ligands [36].

Epitope peptides presented by HLA class I play an important role in the interaction between KIRs and HLA class I ligands [37, 38]; small changes in the peptide sequence can profoundly influence the binding affinity of peptide-HLA complex (pHLA) to KIRs and consequently regulate NK cell responses to the potential target cells [1-4, 39, 40] . In the case of viral infection, the peptide-"specificity" protects uninfected cells (which present self-peptide) from the lysis of NK cells and in the meantime confers the eradication of infected cells (which present viral peptides) by NK cells (Fig. E). Studies on HIV-1 suggested that distinct HIV-1 derived peptides differentially influenced the interaction between KIRs and pHLA complex which consequently resulted in different levels of NK cell activity toward the target [41-43]. The peptide-"specificity" also provides an additional mechanism for the virus to escape from NK cell-mediated immune responses: mutations within epitope peptide sequences which lead to increased binding affinity of the pHLA complex to inhibitory KIRs and to stronger inhibition of NK cells can result in the escape of the mutant virus from NK cell responses [36, 41]. However, the correlation between CTL-selected escape mutations and the putative NK cell-selected mutations remains unclear.

5-4) HLA-C alleles and KIR2D

HLA-C alleles were believed to play a less important role as compared with HLA-A or HLA-B alleles in the defense of viral infection since they have the least expression level on the cell surface. However, Apps et al. reported that increasing HLA-C expression is independently associated with protection against multiple outcomes of HIV-1 infection [44]. This study combined with several others [45-48] suggested the importance of HLA-C restricted CTLs in the

eradication of HIV-1 infection. Moreover, HLA-C alleles which serve as the ligands for KIR2D also largely involve in the manipulation of NK cell function under viral infection. Khakoo et al. showed that KIR2DL3^{+} individuals homozygous for HLA-C1 (HLA-C alleles with asparagine on position 80) had better clinical outcomes [9] suggesting the synergistic protective effect of the KIR2D and HLA-C combination on the eradication of viral infection. NK cell function is largely regulated by the interplay between KIRs and their HLA ligands; both the surface expression of HLA ligands and the peptide-HLA-KIR binding influence NK cell activity [28]. Unlike HLA-A and HLA-B alleles, the surface expression level of HLA-C alleles is not down-regulated by Nef (although a recent study reported that HLA-C expression level can be down-regulated by Vpu from some HIV-1 strains [49]) and thus remains relatively stable after HIV-1 infection [50]. Therefore, the interaction between peptide-HLA-C complex and KIR2D which is sensitive to the sequence variation within the binding peptide might be a key factor in the regulation of anti-viral activity and function of NK cells expressing KIR2D [36, 41, 43].

Indeed, it has been recently reported that KIR2DL2, a B haplotype-specific inhibitory receptor largely involved in the recognition of virus-infected target cells by NK cells [36, 43, 51, 52]. Both *in vitro* and *in vivo* screenings discovered that the independent presence of KIR2DL2 or co-presence of several KIR2DL2/HLA-C1 (HLA-C alleles with asparagine at position 80 which forms the C1 epitope) combinations selected sequence variations in HIV-1 genome [36, 51]. These mutations confer stronger binding of KIR2DL2 to the peptide-HLA complex and result in the inhibition of anti-viral function of KIR2DL2 positive NK cells and the consequent escape of virus-infected target cells from NK cell-mediated killing activity [36, 51]. These studies might indicate the strong immune pressure from KIR2DL2 positive NK cells; however, no direct evidence has been found yet to support KIR2DL2 as a protective KIR gene in HIV-1 infection.

5-5) Aims of the study

a. To determine the frequency and distribution of each KIR gene in the Japanese population;

b. To identify KIR genes or KIR-HLA combinations with protective effect on HIV-1 infection in the Japanese population;

c. To investigate the effect KIR- peptide-HLA interaction on NK cell anti-viral functions;

d. To clarify the mechanisms of protective KIRs or KIR-HLA combinations in HIV-1 infection.

6. Materials and Methods

6-1) Ethics statement

The study was approved by the Ethics Committees of Kumamoto University (RINRI-540 and GENOME-210) and the National Center for Global Health and Medicine (NCGM-A-000172-00). The written informed consent was obtained from all individuals according to the Declaration of Helsinki.

6-2) Study subjects

504 treatment naïve HIV-1 chronically infected individuals and 4 HIV-1 seronegative individuals were recruited. Their plasma and PBMCs were separated from whole blood. HLA genotypes of the HLA-A, B and C allele were identified by the Luminex microbead method at the NPO HLA laboratory (Kyoto, Japan).

6-3) KIR typing

The low-resolution KIR typing which is aimed to detect the presence or absence of 16 KIR genes was performed by using multiplex PCR-SSP [16]. Based on the two haplotypes, individuals were classified as follow: A/A: individuals without any of the haplotype B-specific genes (or we can say individuals having only 3DL3, 2DL3, 2DP1, 2DL1, 2DL1, 3DP1, 2DL4, 3DL1, 2DS4 and 3DL2); A/B: individuals possessing all the haplotype A-specific genes (2DL3, 3DL1 and 2DS4) and at least one haplotype B-specific gene (2DS2, 2DL2, 2DL5, 2DS5 3DS1, 2DS3 and 2DS1); B/B: individuals who lacks some of the haplotype A-specific genes (especially 2DS4).

6-4) NK cell sorting

Cryopreserved PBMC samples from four healthy donors (all had KIR2DL3 whereas three had KIR2DL2/S2 but one did not) were thawed, divided and immediately stained with anti-CD3 mAb, anti-CD16 mAb, anti-CD56 mA, GL183 anti-CD158b mAb (BD Pharmingen™) and 7AAD (BD Pharmingen™). CD158b is the cell surface marker for KIR2DL2, KIR2DS2 and KIR2DL3 which share similar extracellular structure. One thousands of CD3-CD16+CD56+CD158b+7AAD- cells were sorted into a 96-well plate by using a FACSAria I (BD Biosciences). Sorted NK cells from 2DL2/S2+ or 2DL2/S2- donors were stained with anti-KIR2DL3 mAbs (R&D Systems) and the frequency of KIR2DL3+ cells was measured by flow cytometry. NK cells from 2DL2/S2+ donors contained only 4.1-5.9% KIR2DL3+ cells, whereas all of NK cells from 2DL2/S2- donors expressed KIR2DL3. Sorted cells were cultured in cellgroSCGM serum-free medium (CellGenix) supplemented with 10% FBS and 400U/ml rIL-2 for 1-2 months. These cultured NK cells were tested for the expression of CD158b and all of them expressed CD158b. The reactivity of the cultured NK cells were tested against K562 cells by measuring the frequency of NK cells producing IFN-□ or expressing CD107a. They were 99.3 %-100 %.

6-5) Target cell lines

721.221-C1202, -C1402 and C-1403 cell lines were generated by the transfection of HLA-C*12:02, -C*14:02 and -C*14:03 genes into 721.221-CD4 cell lines, respectively. RMA-S-C1202, -C1402 and -C1403 cell lines were generated by the transfection of HLA-C*12:02, -C*14:02 and -C*14:03 genes into RMA-S cell lines, respectively. These cell lines were maintained in RPMI 1640 medium supplemented with 5% FBS (R5) and 0.15 mg/ml

hygromycin B. RMA-S cell line is a TAP2 defected cell line derived from mice. They express high levels of empty MHC molecules (i.e. without binding peptide) on the cell surface when cultured at 26 ℃ and extremely low levels when cultured at 37 ℃ [53].

6-6) HIV-1 clones.

The proviral clones NL4-3 [54] and the chimeric virus clone NL4-3gagHXB2 [55] were previously described. These two clones were used as wild type (WT) virus controls. The NL4-3pol 464-10-9A (9A mutant virus) was generated by introducing the Pol463-10-9A mutation into NL-432 using site-directed mutagenesis (Invitrogen) as previously described [45].

6-7) Viral suppression assay

The ability of sorted NK cell lines to suppress HIV-1 replication was examined as previously described [45, 56]. Briefly, target cells (CD4 T cells or 721.221 cell lines) were incubated with a given HIV-1 clone for 6 h at 37 ℃ after 30min centrifugation with 1000 rpm at 4 ℃. After 3 times wash by R10, the infected cells were co-cultured with different sorted NK cell lines (E:T ratio=0.1:1). From day 4 to day 7 post infection, 30 ul of supernatant was collected and stored at -30℃; and the concentration of p24 Ag was measured by p24 ELISA by using HIV-1 p24 Ag ELISA kit (ZeptoMEtrix). The percentage of suppression of HIV-1 replication was calculated as follow: % suppression= (1-concentration of p24 Ag in the supernatant of HIV-1-infected target cells co-cultured with an NK cell line/ concentration of p24 Ag in the supernatant of HIV-1-infected target cells without effectors) ×100.

6-8) NK cell stimulation and reactivation assay

The activity of NK cells under the stimulation of HIV-1infected target cells or RMA-S cells pre-pulsed with a specific peptide were evaluated by measuring NK cell degranulation and intracellular cytokine staining assay as previously described [34, 57].

For HIV-1 infected target cells, 721.221 cell lines were infected with specific HIV-1 strains for 3 days; viral infectivity were measured by intracellular p24 staining (Beckman Coulter); target cells with similar frequency of p24 positive populations were selected and co-cultured with a specific NK cell line (E/T ratio=0.1:1) for 1 day; Brefeldin A (6ug/ml) and anti-CD107a mAb (FITC) were added 5 h before the termination of co-culture; NK cells were then stained and analyzed by FACS (FACSCanto II).

For peptide-pulsed RMA-S cells, RMA-S cell lines were pre-cultured at 26 ℃ for 18h and then incubated with different concentrations of a specific synthesized peptide (100uM, 30uM and 10uM) at same temperature for 1h; cells were then co-cultured with a specific NK cell line (E/T ratio=0.1:1) for 1h at 37 ℃; Brefeldin A (6ug/ml) and anti-CD107a mAb (FITC) were added and cells were cultured for another 5h; NK cells were then stained and analyzed by FACS (FACSCanto II).

In cell staining, cells were stained with anti-CD56 mAb (PE), anti-CD158b mAb (FITC) and 7AAD, washed with PBS supplemented with 2% FBS, fixed with 4% of paraformaldehyde and permeabilized with PBS-FBS supplemented with 0.1% Saponin; cells were then stained with anti-IFN-γ mAb (Pacific Blue), washed with PBS-FBS supplemented with 0.1% Saponin and

fixed with 2% of paraformaldehyde. CD107a/IFN-γdouble positive and single positive $7AAD^-$ $CD56^+CD158b^+$ cells were all counted as activated NK cell population. For HIV-1 infected target cells, relative NK cell activation (%) was calculated as follow: % of activated NK cells co-cultured with HIV-1-infected target cell lines – % of activated NK cells co-cultured with uninfected target cell lines. For peptide-pulsed RMA-S cells, NK cell activation index was calculated as follow: (% of activated NK cells co-cultured with peptide-pulsed RMA-S cell lines- % of activated NK cells without target cells) / (% of activated NK cells co-cultured RMA-S cell lines without peptide pulsing - % of activated NK cells without target cells).

6-9) Peptide-HLA binding assay

The binding of peptides to HLA class I molecules were measured as previously described [58]. Briefly, RMA-S cell lines were pre-cultured at 26 ℃ for 18 h and then incubated with different concentrations of peptide (from 0uM to 300uM) at same temperature for 1h; cells were then incubated at 37 ℃ for another 3h. After incubation, cells were stained with anti-HLA class I α3 domain mAb TP25.99 [59] and subsequently with FITC-conjugated sheep IgG (Jackson ImmunoResearch Laboratories). The M.F.I. was measured by flow cytometry (FACSCanto II). The normalized HLA expression index was calculated as follow: (MFI of RMA-S cells pre-pulsed with peptide –MFI of RMA-S cells without peptide pulsing) / (MFI of RMA-S cells kept at 26℃ indefinitely – MFI of RMA-S cells without peptide pulsing).

6-10) Peptide-HLA complex and KIR binding assay

HLA-C*12:02, HLA-C*14:02 and HLA-C*14:03 monomers were made as previously described [60], respectively. HLA-C*12:02 monomers were folded with the following peptides: ILKEPVHGVY (WT Pol-IY10) and ILKEPVHGAY (9A Pol-IY10), respectively. HLA-C*14:02 and C*14:03 monomers were folded with the peptide LYNTVATL (Gag-LL8). Surface plasmon resonance studies were performed by using a BIAcore3000 (GE Healthcare) as previously described [41, 61]. Experiments were performed at 25 ℃. HBS-EP buffer (GE Healthcare) was used as the running buffer.

KD values and kinetic were measured either by Scatchard plots or by curve fitting of the data to the Langmuir binding isotherm. All analysis was performed by using BIAevaluation software (version 4.1.1; GE Healthcare) and graphs were made by using Origin software (version 7; Microcal Software, Northampton, Massachusetts, USA).

6-11) Statistical analysis

We performed statistical analyses by using Prism (Graphpad Software) and Origin Software. For the comparison between two groups, we used Mann-Whitney U test. For the comparison between more than three groups, we used Kruskal-Wallis test. We performed logistical regression model with interaction term to analyze the synergistic effect of KIR/HLA combinations on pVL.

7. RESULTS

7-1) The synergistic effect of KIR2DL2/S2 with HLA-C*12:02 or HLA-C*14:03 on HIV-1 pVL in chronically HIV-1 infected Japanese individuals

We recruited 504 ART-naïve Japanese individuals chronically infected with HIV-1. In order to determine the frequency and distribution of each KIR gene in our cohort, we first performed KIR typing by using multiplex PCR-SSP (sequence specific primers set). The result showed that the 4 frame genes (3DL3, 3DL2, 3DP1 and 2DL4) and the two pseudo genes (2DP1 and 3DP1) were presented in 100% of the individuals. Also, haplotype A-specific genes, 2DL3, 3DL1 and 2DS4, showed extremely high frequency among our cohort (**Fig. 1**). These results were consistent with the ones previously reported from Japanese and Caucasians (Allele Frequency Net Database: http://www.allelefrequencies.net/default.asp). However, haplotype B-specific genes (2DS2, 2DL2, 2DS3, 2DS1, 2DS5, 3DS1 and 2DL5) showed significantly lower frequencies compared with haplotype A-specific genes. In addition, 2DS2, 2DL2 and 2DS3 in the two Japanese cohorts showed significantly lower frequencies as compared with the USA Caucasian cohort ($p<0.001$) (**Fig. 1**).

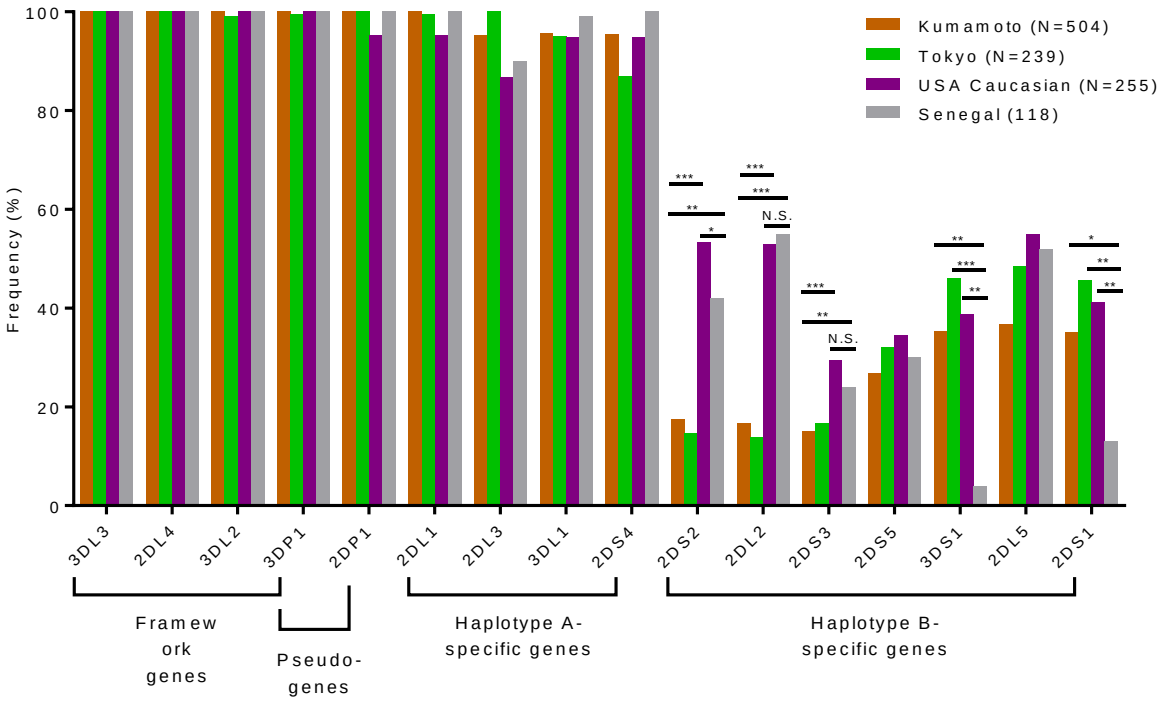


Figure 1. The frequency of KIR genes among different ethnic groups. The presence or absence of each specific KIR gene was analyzed by using low-resolution KIR typing (multiplex PCR-SSP). The data of Tokyo[62], USA Caucasian[63] and Senegal[64] cohorts were obtained from The Allele Frequency Net Database: http://www.allelefrequencies.net/default.asp. P values were calculated by two-tailed Fisher's exact test. **, p<0.01; ***, p<0.001.

To assess the influence of each KIR gene on the HIV-1 infection in our cohort, we next performed statistical analysis to investigate the correlation between the presence of the KIR gene and plasma viral load (pVL). We first analyzed the influence of the KIR haplotypes. We divided the patients into three groups, haplotype A homozygous (AA), haplotype A and B heterozygous (AB) and haplotype B homozygous (BB) and analyzed the correlation between haplotypes and pVL. The result showed that the existence of haplotype B-specific genes had weak beneficial effect on the control of HIV-1 as reflected by significantly lower pVL (*p=0.02884*) (**Fig.2**).

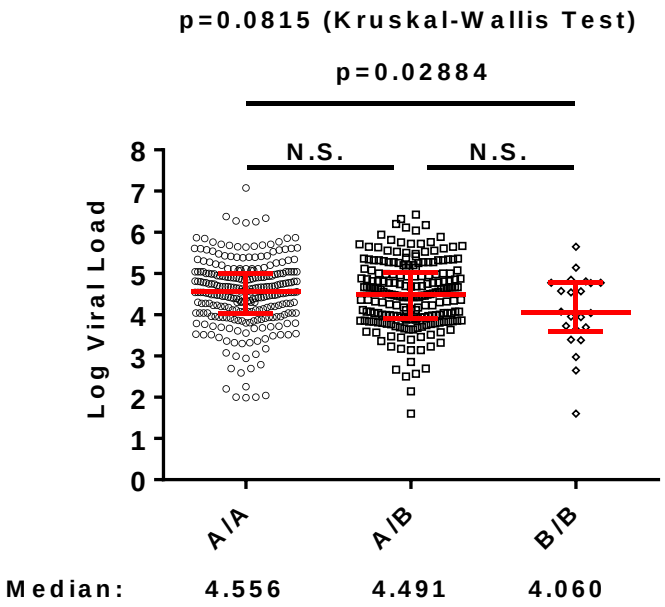


Figure 2. The effect of B haplotype KIR homozygote on pVL. Based on the two haplotypes, individuals were classified as follow: A/A: individuals without any of the haplotype B-specific genes (or we can say individuals having only 3DL3, 2DL3, 2DP1, 2DL1, 2DL1, 3DP1, 2DL4, 3DL1, 2DS4 and 3DL2); A/B: individuals possessing all the haplotype A-specific genes (2DL3, 3DL1 and 2DS4) and at least one haplotype B-specific gene (2DS2, 2DL2, 2DL5, 2DS5 3DS1, 2DS3 and 2DS1); B/B: individuals who lacks some of the haplotype A-specific genes (especially 2DS4). All results are given as median and interquartile range. P values between 2 groups were calculated by Mann-Whitney U test.

Also, since haplotype B-specific genes had significantly lower frequencies (**Fig. 1**), we next focused on the effect of 7 haplotype B-specific KIR genes (2DS2, 2DL2, 2DS3, 2DS5, 3DS1, 2DL5, and 2DS1), analyzing the correlation between the presence of each gene and pVL. The result showed that all the 7 haplotype B-specific genes had no direct beneficial effect on pVL in our cohort though 2DS2 and 2DL2 genes showed a trend of beneficial effect on pVL (**Table 1**). These results together indicated that the sole presence of each KIR gene had no direct effect on HIV-1 infection in our cohort.

Table 1. The effect of each Haplotype B-specific KIR gene on the clinical outcome of HIV-1 infection.

KIR genes	KIR+			KIR-			P value
	N	Median pVL	IQR	N	Median pVL	IQR	
2DS2	90	4.278	3.966-4.975	414	4.544	3.959-5.000	**0.05285**
2DL2	85	4.255	3.973-4.982	419	4.544	3.955-5.000	**0.05988**
2DS3	76	4.431	3.895-5.368	428	4.544	3.961-5.000	0.27705
3DS1	175	4.519	4.041-4.965	329	4.544	3.953-5.000	0.79009
2DS1	174	4.505	3.898-5.556	330	4.544	3.970-4.998	0.43037
2DS5	134	4.531	3.398-4.540	370	4.544	4.000-5.000	0.59187
2DL5	183	4.477	3.997-5.108	321	4.544	3.954-5.000	0.16558

P values were calculated by Mann-Whitney U test.

We then focused on the co-effect of KIR and their HLA ligands on the pVL. We first analyzed co-effect of 3DS1 and HLA-Bw4 80I on pVL since this KIR-HLA combination was one of the most well-known ones with protect effect on HIV-1 infection in Caucasian cohorts [35, 65]. However, our results showed no such significant effect among the entire Bw4-80I group and KIR3DS1 on pVL (**Fig. 3**). We next screened each of the HLA-B alleles belonging to the Bw4-80I group (B*51:01 and B*52:01, respectively) analyzing their co-effect with KIR3DS1 on pVL. The result still showed no significant effect on pVL among the two combinations (**Table 2**).

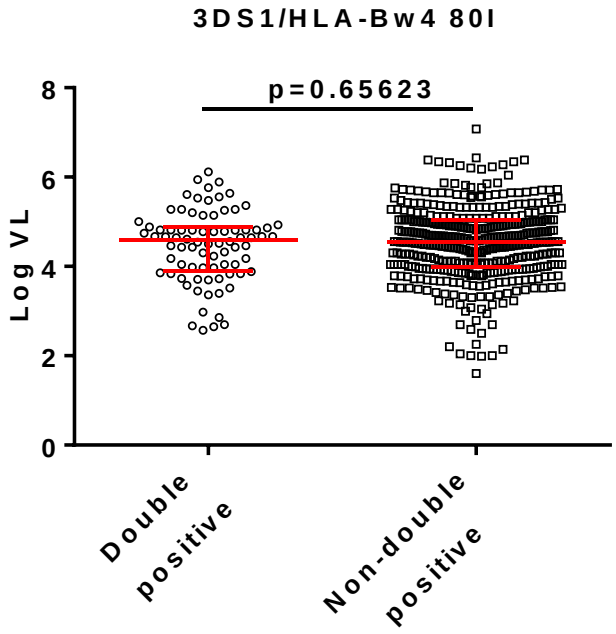


Figure 3. The co-effect of HLA-Bw4 80I and KIR3DS1 on pVL. The results are given as median and interquartile range. P values were calculated by Mann-Whitney U test.

Table 2. The synergistic effect of HLA-Bw4 80I and KIR3DS1 on pVL.

HLA-B alleles	Double Positive			Non-double positive			P value
	N	Median VL	IQR	N	Median VL	IQR	
5101	28	4.667	4.041-5.110	476	4.544	3.955-5.000	0.62464
5201	47	4.431	3.724-4.813	457	4.544	4.000-5.041	0.0666

P values were calculated by Mann-Whitney U test.

We next investigated another KIR-HLA combination, KIR2DL2 and HLA-C1 group. Based on the difference of the amino-acid sequence on the 77th and 80th position on the class I heavy

chain, HLA-C alleles can be divided into two allotypes: HLA-C1 (S/N) or HLA-C2 (N/K) [66] (**Fig. 4A**). Previous studies showed that KIR2DL2 had higher affinity to HLA-C1 than that to HLA-C2, conferring stronger interactions between KIR2DL2 and HLA-C1 [67, 68]. We first analyzed the effect of the independent presence of HLA-C1 group genes on pVL. The result showed that the independent presence of HLA-C1 homozygotes showed significantly lower pVL than other groups including C1/C2 heterozygote and C2 homozygote (**Fig. 4B**). This may result from the effect of the protective allele HLA-C*12:02 which was known in Japanese cohort [69]. We then investigated the effect of co-presence of KIR2DL2 and the entire HLA-C1 group genes. The result showed a trend of beneficial effect on pVL in the KIR2DL2-HLA-C1 double-positive group as reflected by lower pVL although not significant (*p=0.0629*) (**Fig. 4C**).

A

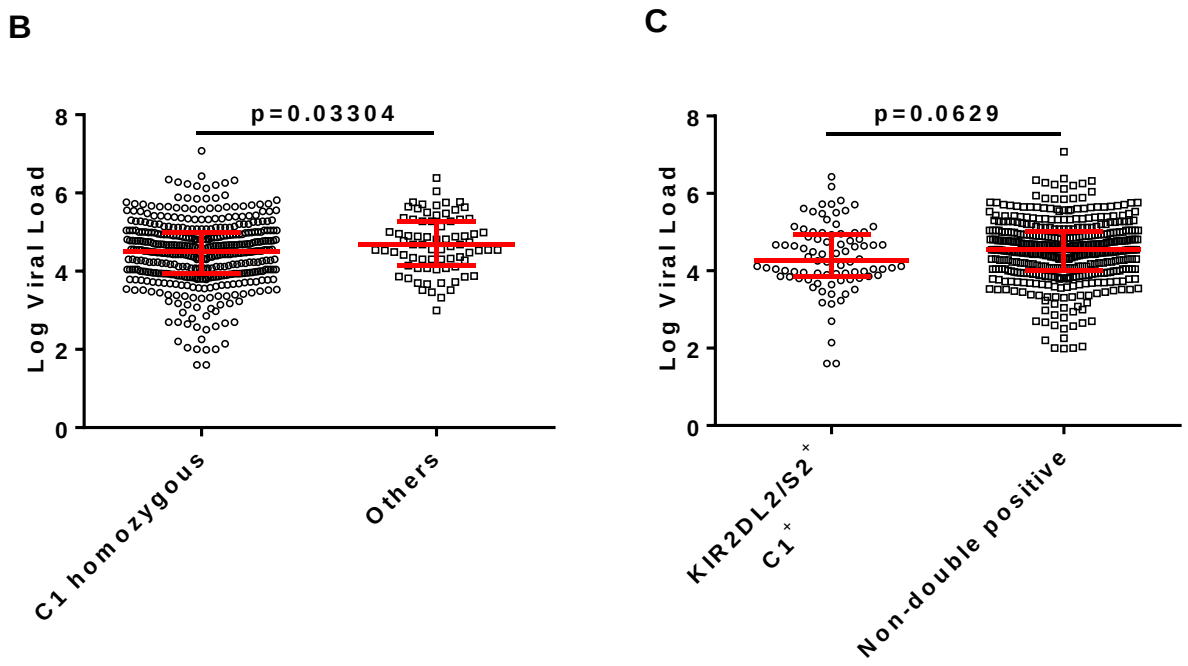


Figure 4. The co-effect of KIR2DL2/S2 and HLA-C1 alleles on HIV-1 pVL. A. HLA-C1 and C2 group genes in our cohort. C1, S/N; C2, N/K. **B.** The effect of HLA-C1 homozygotes on pVL. **C.** The effect of HLA-C1 and KIR2DL2/S2 co-presence on pVL. All results are given as median and interquartile range. P values between 2 groups were calculated by Mann-Whitney U test.

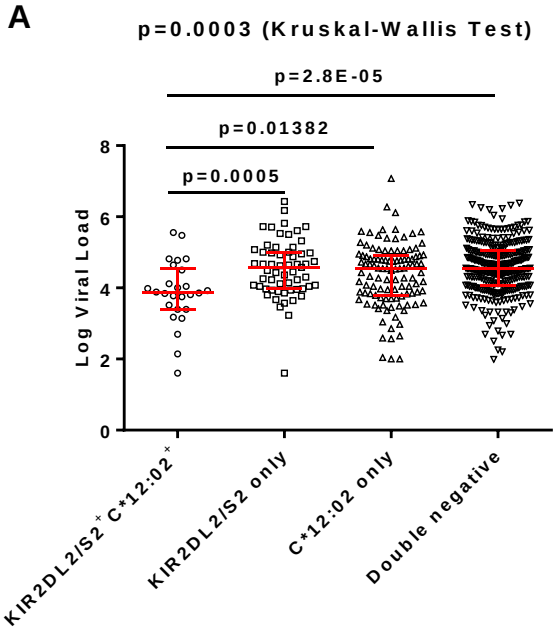
We therefore screened the effect of each C1 group allele (0102, 0303, 0304, 0702, 0801, 1202, 1402 and 1403) with KIR2DL2. Two HLA alleles, HLA-C*12:02 and HLA-C*14:03, showed co-effect with KIR2DL2 on the control of HIV-1 infection as reflected by the significantly lower pVL among double positive individuals (Table 3). To confirm these co-effects, we then further divided the non-double positive individuals into three sub-groups, HLA-C single positive, KIR single positive and double negative. The result showed that individuals carrying KIR2DL2 and HLA-C*12:02 or HLA-C*14:03 showed significantly lower pVL than those having either one or double-negative individuals (**Fig. 5A and 5B**). However, KIR2DL2/S2 did not show such co-

effect with HLA-C*14:02 (**Fig. 5B**) though only one amino acid difference is found at position 21 outside the peptide binding sites between HLA-C*14:02 and HLA-C*14:03.

Table 3. The synergistic effect of KIR2DL2/S2 and HLA-C1 alleles on HIV-1 pVL

HLA-C Alleles	Double Positive			Non-double positive			P value
	N	Median pVL	IQR	N	Median pVL	IQR	
01:02	24	4.506	3.966-4.975	479	4.544	3.959-5.000	0.92849
03:03	19	4.623	3.973-4.982	484	4.538	3.955-5.000	0.97612
03:04	18	4.498	3.895-5.368	485	4.544	3.961-5.000	0.9742
07:02	21	4.643	4.041-4.965	482	4.531	3.953-5.000	0.90032
08:01	11	4.079	3.898-5.556	492	4.544	3.970-4.998	0.83648
12:02	26	**3.872**	3.398-4.540	477	4.544	4.000-5.000	**0.00015**
14:02	12	4.266	3.997-5.108	491	4.544	3.954-5.000	0.84302
14:03	10	**3.835**	3.133-4.237	493	4.544	3.975-5.000	**0.00294**

P values were calculated by Mann-Whitney U test.



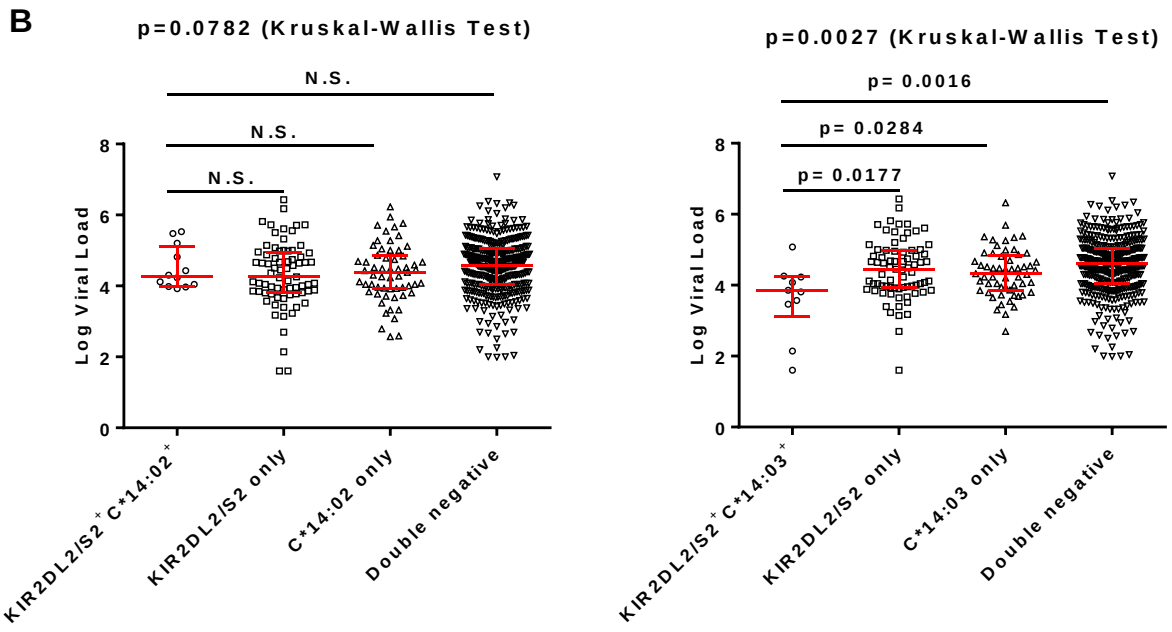


Figure 5. The co-effect of KIR2DL2/S2 and HLA-C*12:02 or HLA-C*14:03 on HIV-1 pVL. **A**. The effect of HLA-C*12:02 and KIR2DL2/S2 co-presence on pVL. **B**. The effect of HLA-C*14:03 or HLA-C*14:02 and KIR2DL2/S2 co-presence on pVL. All results are given as median and interquartile range. P values were calculated by the Mann-Whitney U test or the Kruskal-Wallis test when comparing two or more groups, respectively.

To investigate whether the co-effects of the two KIR/HLA combinations are synergistic or not, we then performed logistic regression model and found that HLA-C*12:02 protection is based on a dual mechanism: one involving NK cell and the other CTL recognition. The protection conferred by HLA-C*14:03, however, is likely due only to its interaction with KIR2DL2/S2 (Table 4).

Table 4. The synergistic effects of KIR/HLA combinations on pVL.

Three variables in each model	N	Mean logVL	SE	P value
KIR2DL2/S2	85	4.21	0.09	0.82
Others	418	4.24	0.10	
HLA-C*12:02	136	4.11	0.09	**0.02**
Others	367	4.33	0.10	
Double positive	26	3.99	0.18	**0.03**
Others	477	4.45	0.06	
KIR2DL2/S2	85	4.02	0.14	0.31
Others	418	4.13	0.14	
HLA-C*14:03	67	3.98	0.14	0.10
Others	436	4.17	0.14	
Double positive	10	3.76	0.27	**0.03**
Others	493	4.40	0.07	

p values were generated by logistical regression model with interaction term.

We also analyzed the effect of other haplotype-B specific KIRs and their HLA ligands on the pVL. The result showed no significant co-effect among these KIR/HLA combinations (Fig. 6A and 6B).

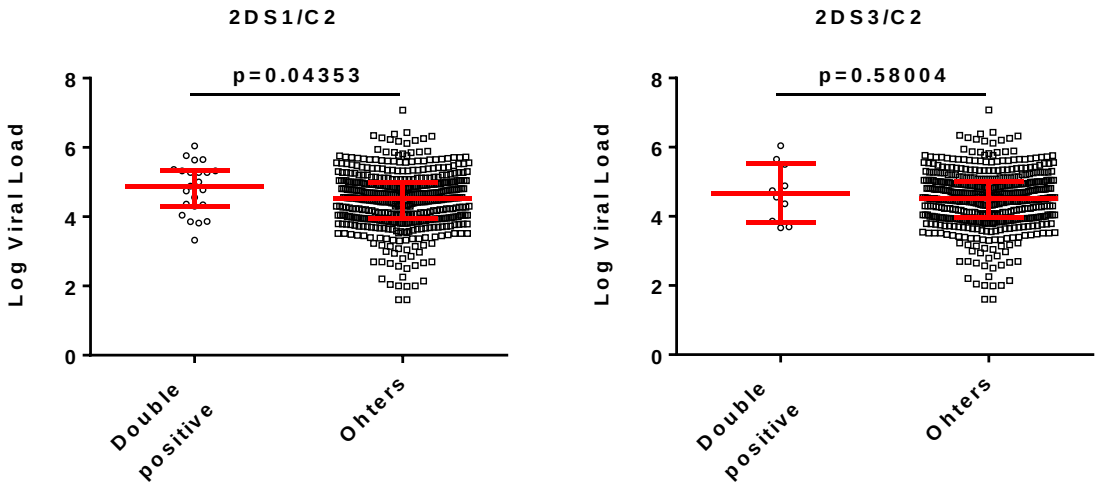


Figure 6. The co-effect of HLA-C2 alleles and KIR2DS1 or KIR2DS3 on pVL. All results are given as median and interquartile range. P values were calculated by Mann-Whitney U test.

7-2) Effective function of KIR2DL2/S2^{+}NK cells for HLA-C*14:03^{+} target cells infected with HIV-1

Based on the results of the genetic analysis, we hypothesized that, (1) In HIV-1-infected HLA-C*14:03^{+} target cells, KIR2DL2/S2^{+} NK cells suppressed viral infection more effectively than that KIR2DL2/S2^{+} NK cells do; (2) KIR2DL2/S2^{+} NK cells suppressed viral infection in HIV-1-infected HLA-C*14:03^{+} target cells more effectively then in HIV-1-infected HLA-C*14:02^{+} cells. In order to test the hypothesis, we first investigated the function of the NK cells on HIV-1-infected HLA-C*14:02^{+} or HLA-C*14:03^{+} cells by viral suppression assay. We established .221 cells transfected with HLA-C*14:02 or HLA-C*14:03 gene (.221-C1402 or .221-C1403) which showed similar HLA-C surface expression levels (Fig. 7A) and performed viral suppression assay by using these .221 cell lines infected with HIV-1 (NL-432gag^{HXB2}) as target cells.

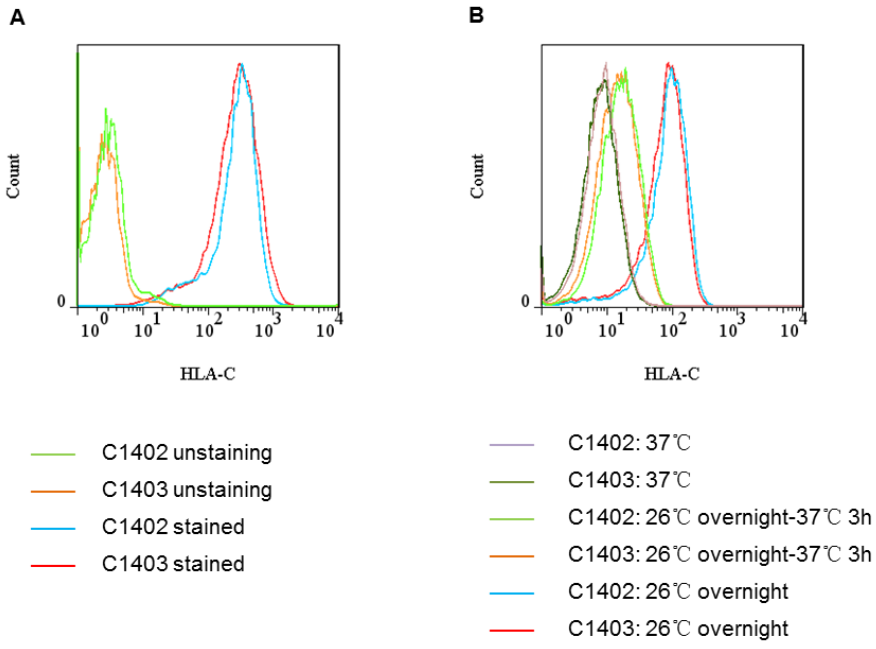
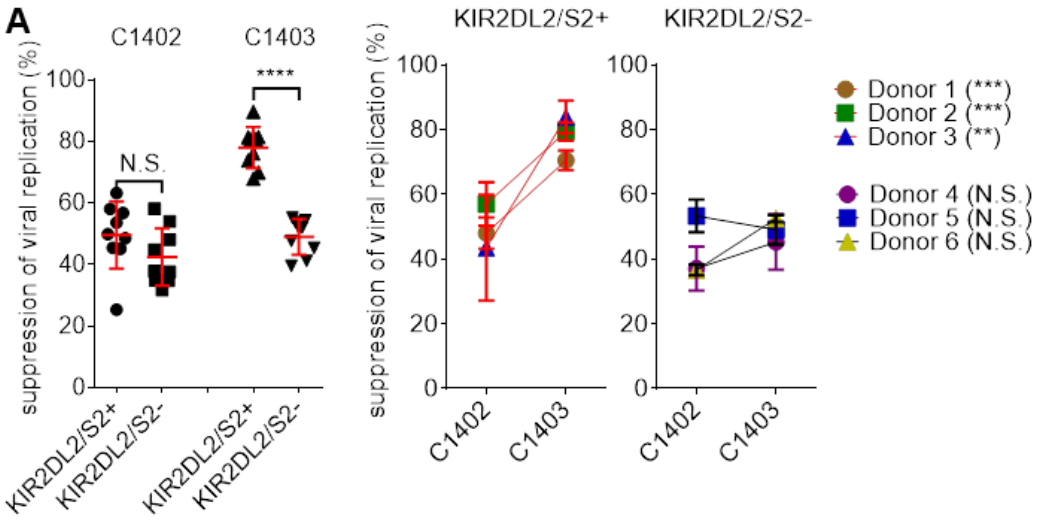


Figure 7. Expression level of HLA-C14 molecules on transfected cells. HLA-C expression level on cell surface of HLA-C*14:02 or –C*14:03 transfectant .221 **(A)** or RMA-S **(B)** cell lines. The HLA expression level was measured by staining the cells with TP25.99 mAb.

KIR2DL2/S2^{+} and KIR2DL2/S2^{-} NK cells (CD3^{-}CD56^{+}) separated from 3 KIR2DL2/S2^{+} healthy donors (Donor 1 to 3) and 3 KIR2DL2/S2^{-} one (Donor 4-6), respectively, were used as effector cells. The results showed that KIR2DL2/S2^{+} NK cells suppressed HIV-1 replication in C1403^{+} cells at significantly higher level than KI2DL2/S2^{-} NK cells; whereas these 2 NK cell lines suppressed HIV-1 replication at the same level in C1402^{+} cells (**Fig. 8A and 8B, left panels**). In addition, 2DL2/S2^{+} NK cells suppressed HIV-1 replication in .221-C1403 cells at significantly higher level than that in C1402+ cells; whereas 2DL2/S2^{-} NK cells suppressed HIV-1 infection in these 2 target cell lines at the same level (**Fig. 8A and 8B, right panels**). We also investigated NK cell activation under the stimulation of .221-C1402 or .221-C1403 cells infected with HIV-1 by measuring IFN-γ secretion and/or CD107a expression. The results exhibited that KIR2DL2/S2^{+} NK cells are more activated under the stimulation of HIV-1-infected .221-C1403 cells than that of .221-C1402 cells (**Fig. 9A**). These results together indicate that KIR2DL2/S2^{+} NK cells more effectively suppress HIV-1 replication in HIV-1-infected C14:03^{+} cells than KIR2DL2/S2^{-} NK cells or in HIV-1-infected C14:02^{+} cells.



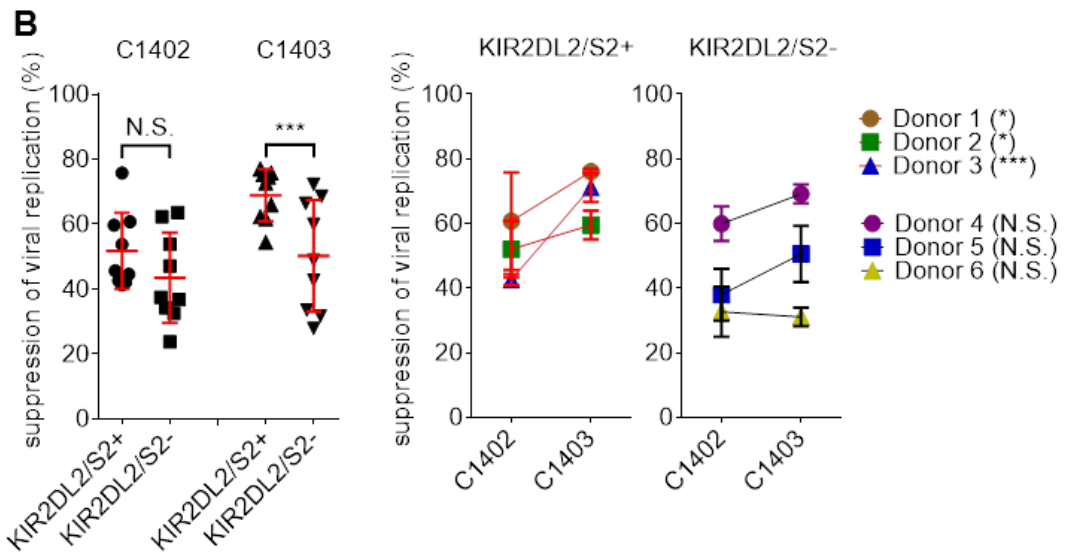


Figure 8. Inhibition of viral replication in HLA-C*14:02 or 14:03 positive target cells by NK cells. A. Normalized percentage of viral suppression in .221-C1402 or .221-C1403 cell lines infected with NL-432gag^{HXB2} by KIR2DL2^{+}/2DS^{+} or 2DL2^{-}/2DS^{-} NK cells. **B.** Normalized percentage of viral suppression in .CD4 T cells separated from HLA-C1402 or -C1403 homozygous individuals infected with NL-432gag^{HXB2} by KIR2DL2^{+}/2DS^{+} or 2DL2^{-}/2DS^{-} NK cells. The left panels show the comparison of the viral suppression level between KIR2DL2/S2^{+} vs. 2DL2/S2^{-} NK cells; the right panels compare the viral suppression level for each individual depending on HLA genotype. *, $p<0.05$; **, $p<0.01$;***, $p<0.001$. P values were calculated by Mann-Whitney U test. All results were acquired by triplicated experiments and were given as mean with SD.

NK cell activity is largely regulated by the interaction between KIR and peptide-HLA complex [28]. To investigate the mechanism of the KIR2DL2/S2^{+} NK cell-mediated viral suppression in HIV-1-infected .221-C1403 cells, we next focused on the interplay between KIR2DL2 and HLA-C*14:02 or -C*14:03 pulsed with an HLA-C14-restricted HIV-1-derived CTL epitope peptide Gag-LL8 [70]. We first established HLA-C*14:02 or HLA-C*14:03-

transfected RMA-S (RMA-S-C1402 or -C1403) cell lines. These two cell lines showed similar HLA-C surface expression level (**Fig. 7B**). By using these two cell lines, we then investigated NK cell activation under the stimulation of HLA-C*14:02 or HLA-C*14:03-transfected RMA-S (RMA-S-C1402 or -C1403) cells pulsed with Gag-LL8 peptide. KIR2DL2^{+} NK cells stimulated with Gag-LL8 peptide-pulsed RMA-S-C14:03 cells showed a significantly higher activation level than those stimulated with RMA-S-C14:02 cells pulsed with the same peptide (**Fig. 9B**).

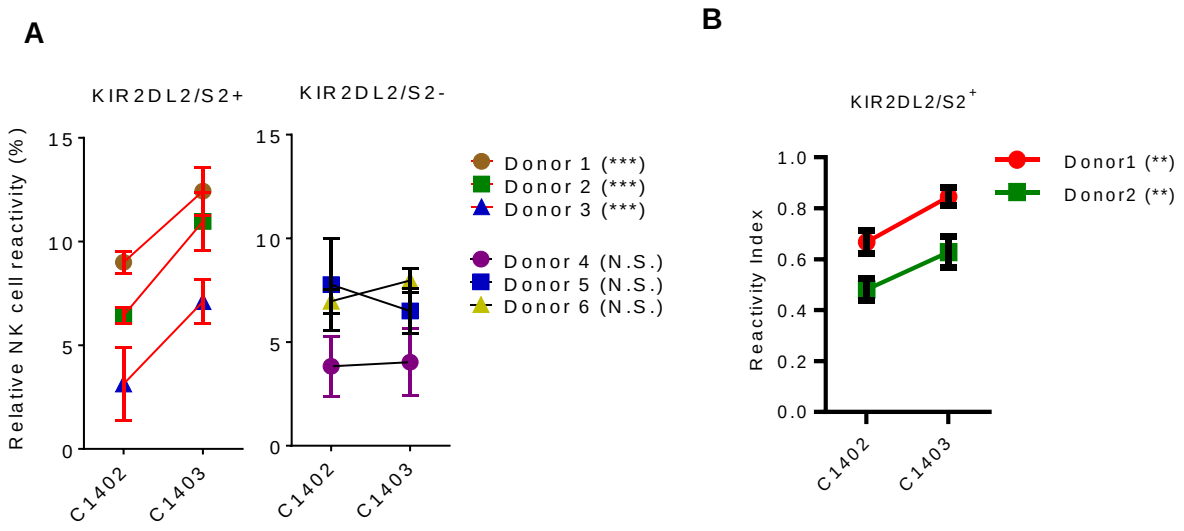


Figure 9. NK cell activation under the stimulation of HLA-C14+ target cells. A. Activation of KIR2DL2^{+}/2DS^{+} or 2DL2^{-}/2DS^{-} NK cells under the stimulation of HIV-1-infected .221-C1402 or C1403 cells. Relative NK cell activation was calculated by using frequency of IFN-γ^{+} and/or CD107a^{+} NK cells as described in materials and methods. **B.** Activation in KIR2DL2^{+}/2DS^{+}NK cells under the stimulation under the stimulation of RMA-S-C1402 or C1403 cell lines pulsed with epitope peptide Gag-LL8 (100 uM). *, $p<0.05$; **, $p<0.01$. P values were calculated by the Mann-Whitney U test. All results were acquired by triplicated experiments and were given as mean with SD.

The above results which showed the difference in NK cell activation level may due to different binding affinity of Gag-LL8 peptide between HLA-C*14:02 and 14:03 molecules which may further confer different surface expression levels of HLA molecules carrying this peptide. To clarify the hypothesis, we first measured the affinity of this peptide to these HLA alleles by HLA stabilization assay using the RMA-S transfectant cells. The result showed that peptide Gag-LL8 stabilized HLA-C*14:02 molecules at significantly higher level than HLA-C*14:03 molecules (**Fig. 10A**), suggesting that the Gag-LL8-HLA-C*14:02 complex had higher surface expression level than –C*14:03 molecules bound with the same peptide.

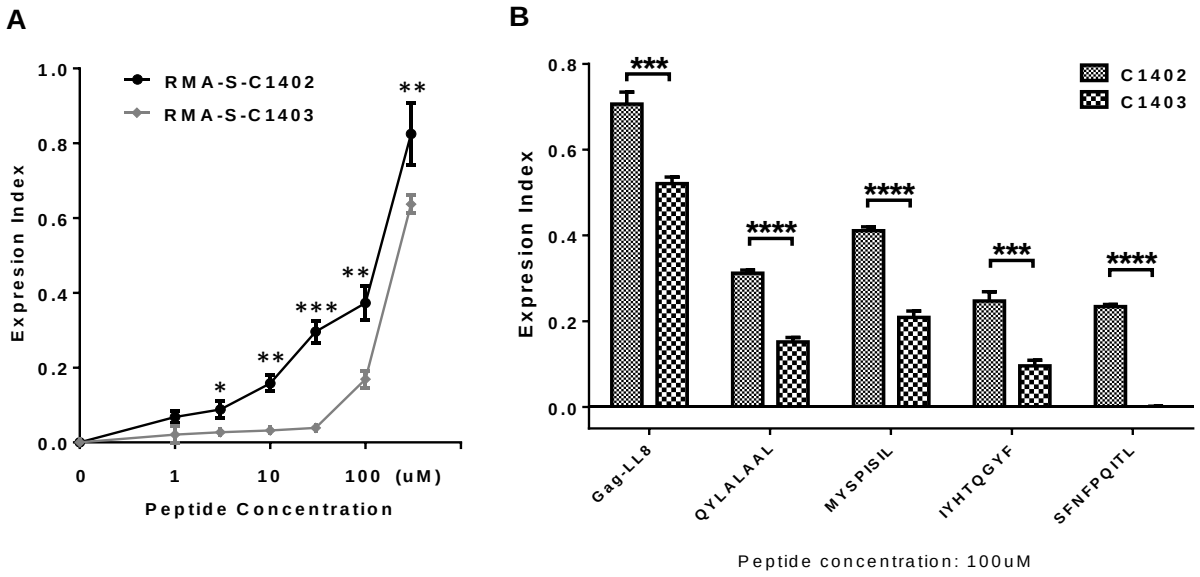
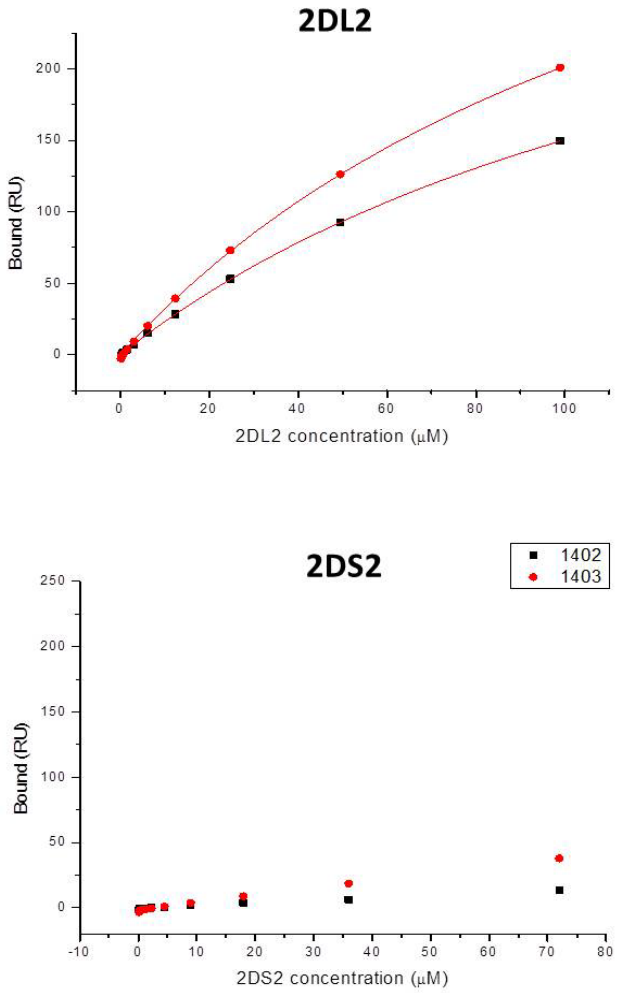


Figure 10. Binding affinity of peptides to HLA-C*14:02 or -C*14:03. A. Normalized expression of HLA molecules on RMA-S-C1402 or -C1403 cell lines pulsed with epitope peptides Gag-LL8. **B.** Normalized expression of HLA molecules on RMA-S-C1402 or C1403 cell lines pulsed with 4 HIV-1 derived peptides (peptide concentration: 100 uM); Gag-LL8 peptide was used as control in the assay. *, $p<0.05$; **, $p<0.01$;***, $p<0.001$; ****, $p<0.0001$. P values were calculated by the Mann-Whitney U test. All results were acquired by triplicated experiments and were given as mean with SD.

To clarify the possibility that the binding affinity of the KIRs affect NK cell function, we directly measured the affinity of KIR2DL2 or KIR2DS2 for Gag-LL8-binding HLA-C*14:02 or Gag-LL8 binding HLAC*14:03 by using BIAcore. The results showed that the binding affinity of KIR2DL2 for Gag-LL8-HLA-C*14:02 was mostly similar to that for Gag-LL8-HLA-C*14:03 whereas KIR2DS2 did not bind to these HLA molecules (**Fig. 11**). These results excluded the possibility that different affinities of KIR2DL2 between these HLA molecules result in the different effect of the NK cells on HIV-1-infected cells. Also, the above results excluded the role of KIR2DS2. Thus, these results together indicate that lower expression of Gag-LL8-HLA-C*14:03 on cell surface of HIV-1-infcted HLA-C*14:03^{+} cells may reduce the inhibition of NK cell recognition via the inhibitory NK cell receptor, KIR2DL2, for Gag-LL8-HLA-C*14:03.



KIR-peptide-HLA	Binding affinity KD (μmol/l)
2DL2-WT-C1402	143
2DL2-WT-C1403	157
2DS2	All no binding

Figure 11. Binding affinity of HLA-C*14:02 or -C*14:03 monomers folded with peptide Gag-LL8 to KIR2DL2 or 2DS2 molecules. The K_D values were determined using equilibrium binding curves and Scatchard analysis of equilibrium binding.

To clarify the affinity of other HLA-C*14 binding HIV-1 peptides between the 2 HLA-C*14 subtypes, we selected 4 HIV-1-derived peptides from HIV-1 peptides carrying HLA-C*14 binding motif. The result showed that all of these 4 peptides bound to HLA-C*14:02 significantly stronger than to HLA-C*14:03 molecules (**Fig. 10B**). The result supported the idea

that HIV-1-derived peptides had stronger affinity to HLA-C*14:02 than HLA-C*14:03 in HIV-1-infected cells.

7-3) Effective function of KIR2DL2/S2^{+}NK cells for HLA-C*12:02^{+} target cells infected with HIV-1

We next investigated the function of KIR2DL2/S2^{+} NK cells for HIV-1-infected HLA-C*12:02^{+} cells by viral suppression assay using .221-C1202 cells infected with HIV-1 (NL-432) as the target. Unlike the KIR2DL2/HLA-C*14:03 combination, KIR2DL2/S2^{+} NK cells did not have significantly higher ability to suppress viral replication than that of KIR2DL2/S2^{-} NK cells (**Fig. 12A and 12B, left panels**), suggesting that the synergistic effect of KIR2DL2/ HLA-C*12:02 combination is due to different mechanism from the case of HLA-C*14. HLA-C*12:02 is a protective allele in HIV-1 infection in the Japanese population [69]. HLA-C*12:02 restricted CTLs can select escape mutations in HIV-1 sequence [45, 71]. We speculated that escape mutations selected by HLA-C*12:02-restricted CTLs may have some effects on KIR2DL2/S2^{+} NK cell recognition. We previously demonstrated that a V to A substitution at Pol 464 which located at the 9th position of an HLA-C*12:02 restricted epitope Pol-IY10 significant impairs the killing activity of Pol-IY10-specific CTLs [45]. To investigate the effect of this 9A-mutation on the suppression of viral replication by NK cells, we performed viral suppression assay on both WT (NL-432) and 9A-mutant viruses (NL-432 with 9A mutation) by using both .221-C1202 or CD4 T cells separated from an HLA-C*12:02 homozygous healthy donor. The results showed that KIR2DL2/S2^{+} NK cells suppressed the replication of the 9A virus more effectively than the WT virus whereas KIR2DL2/S2^{-} NK cells evenly suppressed the replication of 9A and WT viruses (**Fig.12A and 12B, right panels**).

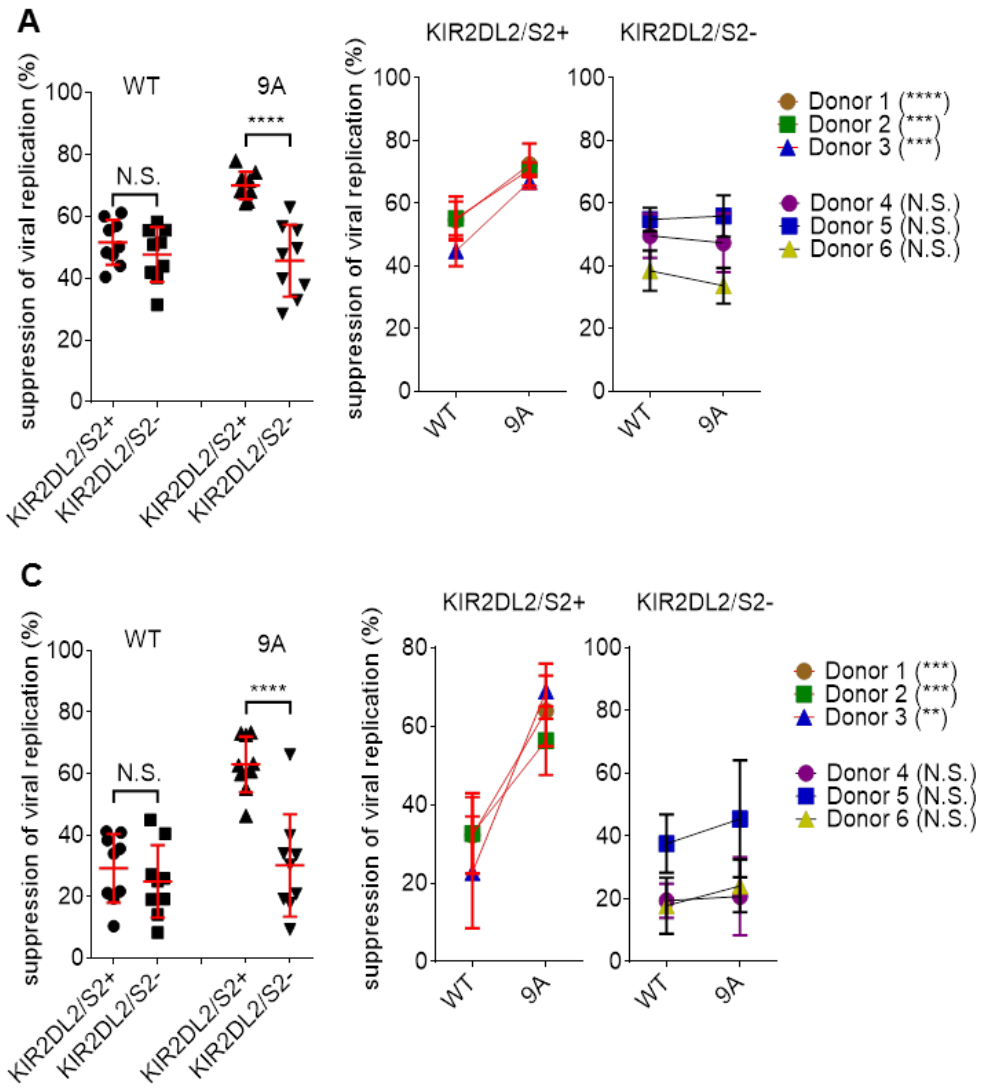


Figure 12. Inhibition of viral replication in HLA-C*12:02 positive target cells infected with WT or 9A mutant virus by NK cells. A. Normalized percentage of viral suppression in .221-C1202 cell lines infected with WT or 9A mutant virus by KIR2DL2^{+}/2DS^{+} or 2DL2^{-}/2DS^{-} NK cells. **B.** Normalized percentage of viral suppression in .CD4 T cells separated from HLA-C1202 homozygous individuals infected with WT or 9A mutant virus by KIR2DL2^{+}/2DS^{+} or 2DL2^{-}/2DS^{-} NK cells. The left panels show the comparison of the viral suppression level between KIR2DL2/S2^{+} vs. 2DL2/S2^{-} NK cells; the right panels compare the viral suppression level for each individual depending on HLA genotype. *, $p<0.05$; **, $p<0.01$;***, $p<0.001$. P values were calculated by Mann-Whitney U test. All results were acquired by triplicated experiments and were given as mean with SD.

We next investigated NK cell activation under the stimulation of .221-C1202 cells infected with the WT or 9A mutant virus. The results exhibited that KIR2DL2/S2^{+} NK cells are more activated by stimulation of the 9A mutant virus-infected .221-C1202 cells than that of KIR2DL2/S2^{-} NK cells or that by WT virus-infected .221-C1202 cells (**Fig. 13A**). To clarify the mechanism of 9A mutation, we then performed NK cell functional assay by using HLA-C*12:02-transfected RMA-S cells (RMA-S-C1202) pulsed with the WT or the mutant peptides. The result showed that RMA-S-C*12:02 cells pre-pulsed with the mutant peptide stimulated KIR2DL2/S2^{+} NK cells at significantly higher level than those prepulsed with the WT peptide (**Fig.13B**), suggesting that the 9A mutation influenced the recognition of the target cells by NK cells via KIR2DL2/S2.

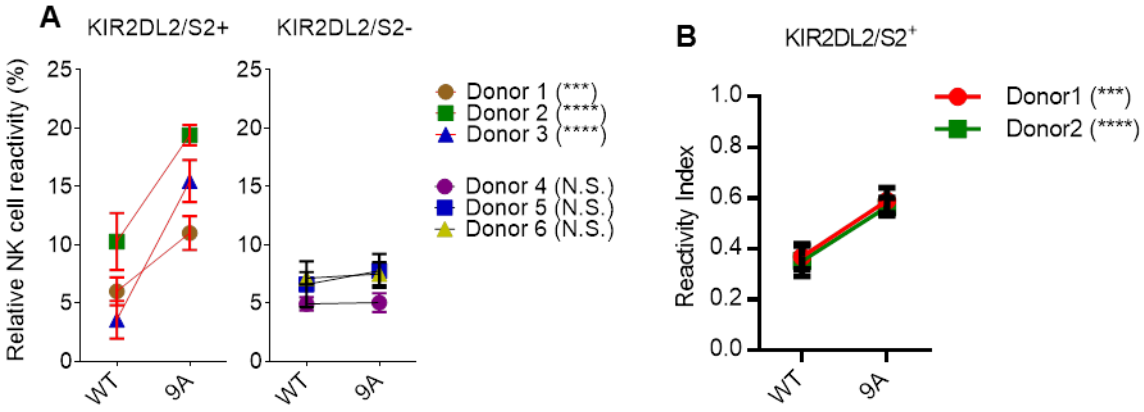


Figure 13. NK cell activation under the stimulation of HLA-C12:02 positive target cells. A. Activation of KIR2DL2^{+}/2DS^{+} or 2DL2^{-}/2DS^{-} NK cells under the stimulation of WT- or 9A mutant virus-infected .221-C1202 cell lines. Relative NK cell activation was calculated by using frequency of IFN-γ^{+} and/or CD107a^{+} NK cells as described in materials and methods. **B.** Activation of KIR2DL2^{+}/2DS^{+} or 2DL2^{-}/2DS^{-} NK cells under the stimulation of RMA-S-C1202 cell lines pulsed with Pol-IY10 or 9A mutant peptide (100 uM). *, $p<0.05$; **, $p<0.01$. P values

were calculated by Mann-Whitney U test.All results were acquired by triplicated experiments and were given as mean with SD.

To clarify how the 9A mutation influences KIR2DL2/S2^{+}NK cell activation, we first measured the binding affinity of these two peptides to HLA-C*12:02 molecules. The result showed that the 9A mutant peptide had significantly weaker binding affinity to the HLA-C*12:02 molecules than the WT peptide (**Fig. 14**), suggesting that the 9A mutation reduced the expression level of HLA-C*12:02 molecules on the 9V mutant virus-infected cells.

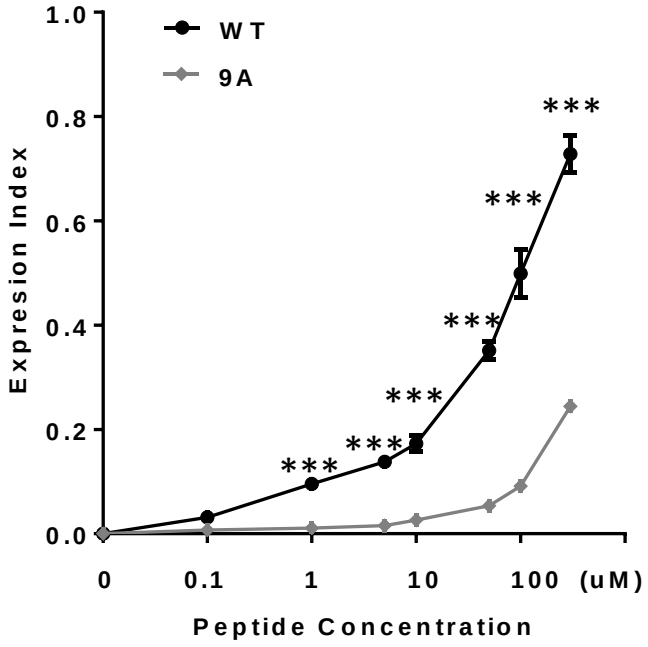
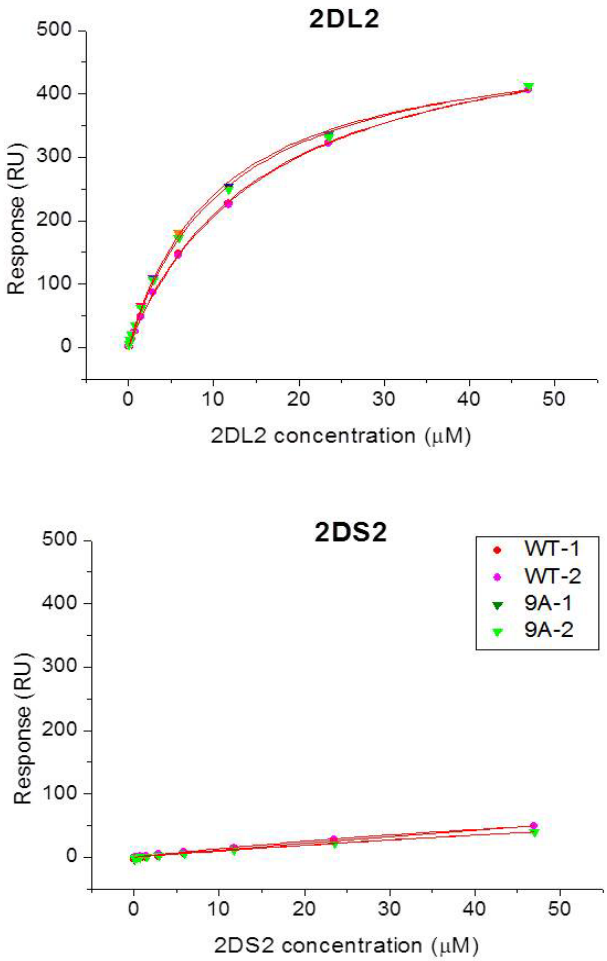


Figure 14. Binding affinity of WT or 9A mutant peptides to HLA-C*12:02. Normalized expression of HLA molecules on RMA-S-C1202 cell lines pulsed with the Pol-IY10 (WT) or 9A mutant peptides. *, p<0.05; **, p<0.01;***, p<0.001; ****, p<0.0001. P values were calculated by Mann-Whitney U test. All results were acquired by triplicated experiments and were given as mean with SD.

We further analyzed a direct binding of KIR2DL2 and KIR2DS2 to HLA-C12:02-peptide complex by using BIAcore. The results showed that the binding affinity of KIR2DL2 to WT Pol-IY10-HLA-C*12:02 was mostly similar to that to mutant Pol-IY10-HLA-C*12:02 complexes, whereas KIR2DS2 did not bind to these HLA molecules (**Fig. 15**). These results indicate that lower expression of mutant Pol-IY10-HLA-C*12:02 on HIV-1-infcted HLA-C*12:02^{+} cells may reduce the inhibition of NK cell recognition via KIR2DL2.



KIR-peptide-HLA	Binding affinity KD (μmol/l)
2DL2-WT-C1202-1	11
2DL2-WT-C1202-2	11
2DL2-9A-C1202-1	11
2DL2-9A-C1202-2	12
2DS2	All no binding

Figure 15. Binding affinity of HLA-C*12:02 monomers folded with WT Pol-IY10 or with 9A-Pol-IY10 peptide to KIR2DL2 or 2DS2 molecules. The K_D values were determined using equilibrium binding curves and Scatchard analysis of equilibrium binding.

8. DISCUSSION

NK cells play a crucial role in the eradication of viral infection; their function is regulated by the interaction between KIRs and the related HLA class I ligands. Previous studies have reported that the presence of some specific KIR genes and/or KIR-HLA gene combinations confers better HIV-1 control [29, 34-36, 43, 51, 65]. These studies were investigated in Caucasian and African cohorts. Since Japanese had different HLA and KIR distribution (Fig. 1), the effect of KIR on viral infection may be different from that in Caucasian and Africans. In the present study, we first performed the genetic analysis for a cohort of 504 treatment naïve HIV-1 chronically infected Japanese patients in order to investigate the influence of KIR genes on the HIV-1 control. We identified two KIR/HLA combinations, KIR2DL2/HLA-C*12:02 and KIR2DL2/HLA-C*14:03, significantly correlated with lower pVL. Previous studies reported the synergistic effect of KIR2DL2 and two HLA-C alleles, HLA-C*01:02 [36, 43] and C*03:04 [51], which belong to the C1 Group on NK cell function and HIV-1 infection in Caucasian cohorts. However, HLA-C*12:02 and C*14:03, which had extremely low frequency in Caucasians, were firstly identified to have synergistic effect with KIR2DL2 in our Japanese cohort with HIV-1 infection. We could not find any protective effect of 3DS1-HLA-Bw4 80I combination, which had been widely reported in the previous studies [34, 35], indicating that the synergistic effect of this KIR-HLA combination in the Japanese population is different from that of Caucasians. Taken together, we identified two new HLA-KIR combinations with protective synergistic effect on the genetic analysis level.

Several previous studies demonstrated that some of the KIR-HLA combinations confer stronger NK cell activity as reflected by the higher level of cytokine secretion and/or stronger cytotoxicity [72-74], which consequently result in the stronger inhibition of viral replication [34,

75]. We initially performed viral suppression assay by using HIV-1 infected .221 cells transfected with different HLA genes as the target and demonstrated that co-presence of KIR2DL2-HLA-C*14:03 confers higher level of IFN-γ secretion and cell degranulation as well as stronger inhibition of HIV-1 replication. Interestingly, HLA-C*14:02 did not show the synergic effect in the genetic analysis as well as *in vitro* viral suppression and NK cell activation assays. HLA-C*14:02 and -C*14:03 share similar sequence with only one amino acid difference on the 21st position located outside of the peptide binding cleft between the 2 molecules. NK cell activation assays by using RMA-S-C1402) or RMA-S-C1403 pre-pulsed with Gag-LL8 epitope peptide demonstrated that KIR2DL2^{+} NK cells stimulated with the peptide-pulsed RMA-S-C1403 cells were activated at significantly higher level than those stimulated with peptide-pulsed RMA-S-C1402 cells. . This was explained by lower expression of HLA-C*14:03 in the cells than that of HLA-C*14:02 due to lower peptide affinity to the former molecule than the later one. However, when checking the direct KIR binding of peptide-HLA complexes, we found that Gag-LL8-HLA-C*14:02 and Gag-LL8-HLA-C*14:03 showed same level of binding affinity to the KIR2DL2 molecule. The peptide binding analysis showed that all of peptides tested had higher affinity to HLA-C*14:02 than HLA-C*14:03. Taken together, these data suggested that single amino acid difference between HLA-C*14:02 and HLA-C*14:03 outside the binding cleft influences the binding affinity of the peptide to the HLA molecules and consequently modulates the recognition of HIV-1-infected target cells by NK cells through KIR2DL2.

Several studies have reported peptide sequence variations may enhance the binding of inhibitory KIRs to the peptide-HLA complexes and consequently down-regulate the activity of NK cells [36, 41, 43, 51]. However, our data showed that a V to A mutation (9A mutation) in an HLA-C*12:02 restricted epitope peptide Pol-IY10 did not influence the direct binding of

KIR2DL2 to the peptide-HLA-C*12:02 complex although it up-regulated KIR2LD2$^+$ NK cell activity and resulted in stronger inhibition of the virus carrying this mutation. The peptide-HLA binding assay showed that the 9A mutation reduced peptide-HLA binding affinity. Taken together, our data suggested that peptide sequence variations influence the peptide-HLA binding affinity which consequently regulates the recognition and activation of NK cells without changing the KIR-pHLA binding affinity.

In the recent years, HIV-1 sequence variations under NK cell mediated immune pressure have been reported [36, 51]. These KIR-correlated sequence variations impair NK cell function by facilitating the binding and recognition of specific HLA alleles to related inhibitory KIRs. On the other hand,, it has been widely reported that strong immune pressure conferred by HIV-1-specific CTLs selects escape mutations within HIV-1 sequence. These mutations result in the evasion of the virus from CTL mediated immunity and consequently accumulate in individuals possessing related HLA alleles [71, 76-78]. However, the impact of these CTL selected escape mutations on KIR recognition and NK cell function had been poorly studied. Thananchai et al. reported that a single amino acid variation within an HLA-Cw4-restricted epitope peptide impaired KIR2DL1$^+$ NK cell function by increasing the direct binding of peptide-HLA complex to KIR2DL1 molecules and also impaired the CTL recognition, implying that CTL escape mutation affect NK cell recognition [41]. We showed that an escape mutation selected by the HLA-C*12:02-restricted CTLs, which accumulated in HLA-C*12:02$^+$ individuals without reversion (Fig 15) [45], activated the KIR2DL2$^+$ NK cells, so that they suppressed mutant virus replication in the meantime. These findings suggested that the 9A mutation may influence the recognition of 2DL2$^+$ NK cells at population level and contribute to the protective synergistic effect of KIR2DL2/HLA-C*12:02 combination on HIV-1 infection.

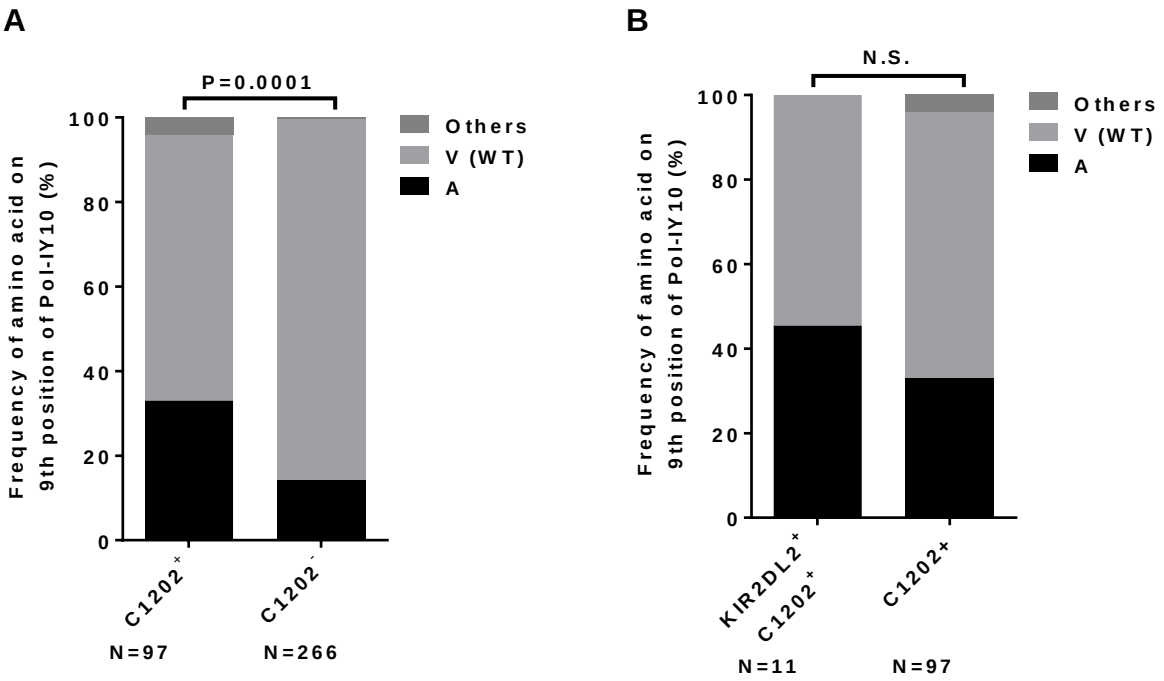
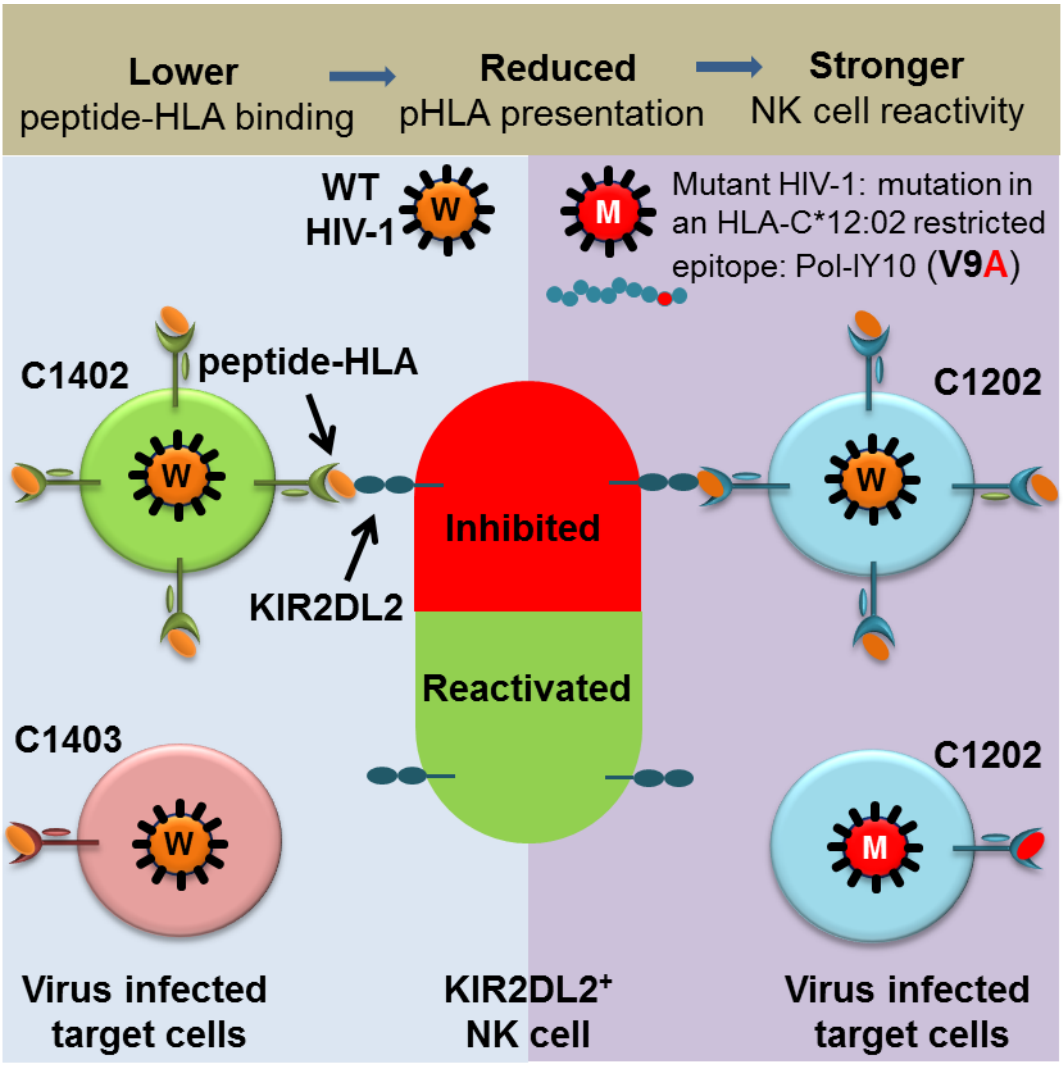


Figure 15. The 9A mutation in peptide Pol-IY10. A. The frequency of each amino acid on the 9th position of Pol-IY10 among HLA-C*12:02^{+} and HLA-C*12:02^{-} individuals. **B.** The frequency of each amino acid on the 9th position of Pol-IY10 among KIR2DL2^{+}HLA-C*12:02^{+} and HLA-C*12:02^{+} individuals. P value was calculated by two-tailed Fisher's exact test.

9. CONCLUSION

NK cells play a crucial role in both innate immune system and acquired immune system in the eradication of viral infection, including HIV-1 infection. The function and activity of NK cells is largely regulated by the interaction between KIR and peptide-HLA complex. In the recent years, the effect of KIR, whether independent or combined with their HLA ligands, on HIV-1 infection has been widely reported.



In the present study, we performed a comprehensive study on the synergistic effect of KIR-HLA combinations in the Japanese population which had never been studied and identified two new combinations, KIR2LD2/HLA-C*12:02 and KIR2LD2/HLA-C*14:03, having protective effect on HIV-1 control. Although the following in vitro study only focused on limited number of epitope peptides, we initially gave new insights to the peptide-HLA binding and NK cell recognition and function. Moreover, we investigated the impact of CTL selected escape mutations on KIR recognition and NK cell activation in the first time uncovering the delicate network between acquired immune system and innate immune system under HIV-1 infection.

10. REFERENCE

1. Malnati, M.S., et al., *Peptide specificity in the recognition of MHC class I by natural killer cell clones.* Science, 1995. **267**(5200): p. 1016-8.
2. Peruzzi, M., et al., *Peptide sequence requirements for the recognition of HLA-B*2705 by specific natural killer cells.* J Immunol, 1996. **157**(8): p. 3350-6.
3. Mandelboim, O., et al., *Self and viral peptides can initiate lysis by autologous natural killer cells.* Proc Natl Acad Sci U S A, 1997. **94**(9): p. 4604-9.
4. Hansasuta, P., et al., *Recognition of HLA-A3 and HLA-A11 by KIR3DL2 is peptide-specific.* Eur J Immunol, 2004. **34**(6): p. 1673-9.
5. Sun, J.C., J.N. Beilke, and L.L. Lanier, *Adaptive immune features of natural killer cells.* Nature, 2009. **457**(7229): p. 557-61.
6. Romee, R., et al., *Cytokine activation induces human memory-like NK cells.* Blood, 2012.
7. Sun, J.C., et al., *NK cells and immune "memory".* J Immunol, 2011. **186**(4): p. 1891-7.
8. Heim, M.H. and R. Thimme, *Innate and adaptive immune responses in HCV infections.* J Hepatol, 2014. **61**(1S): p. S14-S25.
9. Khakoo, S.I., et al., *HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection.* Science, 2004. **305**(5685): p. 872-4.
10. Braun, M., et al., *NK cell activation in human hantavirus infection explained by virus-induced IL-15/IL15Ralpha expression.* PLoS Pathog, 2014. **10**(11): p. e1004521.
11. Yoshimori, M., et al., *CD137 expression is induced by Epstein-Barr virus infection through LMP1 in T or NK cells and mediates survival promoting signals.* PLoS One, 2014. **9**(11): p. e112564.
12. Hendricks, D.W., et al., *Cutting edge: NKG2C(hi)CD57+ NK cells respond specifically to acute infection with cytomegalovirus and not Epstein-Barr virus.* J Immunol, 2014. **192**(10): p. 4492-6.
13. Reilly, E.C., et al., *Neonatal hyperoxia leads to persistent alterations in NK cell responses to influenza A virus infection.* Am J Physiol Lung Cell Mol Physiol, 2014: p. ajplung 00233 2014.
14. Williams, K.J., et al., *VSVDeltaG/EBOV GP-Induced Innate Protection Enhances Natural Killer Cell Activity to Increase Survival in a Lethal Mouse Adapted Ebola Virus Infection.* Viral Immunol, 2014.
15. Uhrberg, M., et al., *Human diversity in killer cell inhibitory receptor genes.* Immunity, 1997. **7**(6): p. 753-63.
16. Kulkarni, S., M.P. Martin, and M. Carrington, *KIR genotyping by multiplex PCR-SSP.* Methods Mol Biol, 2010. **612**: p. 365-75.
17. Anfossi, N., et al., *Human NK cell education by inhibitory receptors for MHC class I.* Immunity, 2006. **25**(2): p. 331-42.
18. Sun, J.C. and L.L. Lanier, *Tolerance of NK cells encountering their viral ligand during development.* J Exp Med, 2008. **205**(8): p. 1819-28.
19. Yawata, M., et al., *MHC class I-specific inhibitory receptors and their ligands structure diverse human NK-cell repertoires toward a balance of missing self-response.* Blood, 2008. **112**(6): p. 2369-80.
20. Hoglund, P. and P. Brodin, *Current perspectives of natural killer cell education by MHC class I molecules.* Nat Rev Immunol, 2010. **10**(10): p. 724-34.

21. Orr, M.T. and L.L. Lanier, *Natural killer cell education and tolerance.* Cell, 2010. **142**(6): p. 847-56.
22. Held, W., et al., *The function of natural killer cells: education, reminders and some good memories.* Curr Opin Immunol, 2011. **23**(2): p. 228-33.
23. Elliott, J.M. and W.M. Yokoyama, *Unifying concepts of MHC-dependent natural killer cell education.* Trends Immunol, 2011. **32**(8): p. 364-72.
24. Sun, J.C. and L.L. Lanier, *Cutting edge: viral infection breaks NK cell tolerance to "missing self".* J Immunol, 2008. **181**(11): p. 7453-7.
25. Carrington, M., M.P. Martin, and J. van Bergen, *KIR-HLA intercourse in HIV disease.* Trends Microbiol, 2008. **16**(12): p. 620-7.
26. Kulkarni, S., M.P. Martin, and M. Carrington, *The Yin and Yang of HLA and KIR in human disease.* Semin Immunol, 2008. **20**(6): p. 343-52.
27. Lanier, L.L., *Up on the tightrope: natural killer cell activation and inhibition.* Nat Immunol, 2008. **9**(5): p. 495-502.
28. Altfeld, M. and P. Goulder, *'Unleashed' natural killers hinder HIV.* Nat Genet, 2007. **39**(6): p. 708-10.
29. Martin, M.P., et al., *Innate partnership of HLA-B and KIR3DL1 subtypes against HIV-1.* Nat Genet, 2007. **39**(6): p. 733-40.
30. O'Connor, G.M., et al., *Functional polymorphism of the KIR3DL1/S1 receptor on human NK cells.* J Immunol, 2007. **178**(1): p. 235-41.
31. Kim, S., et al., *HLA alleles determine differences in human natural killer cell responsiveness and potency.* Proc Natl Acad Sci U S A, 2008. **105**(8): p. 3053-8.
32. Alter, G., et al., *HLA class I subtype-dependent expansion of KIR3DS1+ and KIR3DL1+ NK cells during acute human immunodeficiency virus type 1 infection.* J Virol, 2009. **83**(13): p. 6798-805.
33. Boulet, S., et al., *HIV protective KIR3DL1 and HLA-B genotypes influence NK cell function following stimulation with HLA-devoid cells.* J Immunol, 2010. **184**(4): p. 2057-64.
34. Song, R., et al., *HIV Protective KIR3DL1/S1-HLA-B Genotypes Influence NK Cell-Mediated Inhibition of HIV Replication in Autologous CD4 Targets.* PLoS Pathog, 2014. **10**(1): p. e1003867.
35. Martin, M.P., et al., *Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS.* Nat Genet, 2002. **31**: p. 429 - 434
36. Alter, G., et al., *HIV-1 adaptation to NK-cell-mediated immune pressure.* Nature, 2011. **476**(7358): p. 96-100.
37. Vivian, J.P., et al., *Killer cell immunoglobulin-like receptor 3DL1-mediated recognition of human leukocyte antigen B.* Nature, 2011. **479**(7373): p. 401-5.
38. Mandelboim, O., et al., *The binding site of NK receptors on HLA-C molecules.* Immunity, 1997. **6**(3): p. 341-50.
39. Thananchai, H., et al., *Cutting Edge: Allele-specific and peptide-dependent interactions between KIR3DL1 and HLA-A and HLA-B.* J Immunol, 2007. **178**(1): p. 33-7.
40. Fadda, L., et al., *Peptide antagonism as a mechanism for NK cell activation.* Proc Natl Acad Sci U S A, 2010. **107**(22): p. 10160-5.
41. Thananchai, H., et al., *Reciprocal recognition of an HLA-Cw4-restricted HIV-1 gp120 epitope by CD8+ T cells and NK cells.* AIDS, 2009. **23**(2): p. 189-93.

42. Fadda, L., et al., *Common HIV-1 peptide variants mediate differential binding of KIR3DL1 to HLA-Bw4 molecules.* J Virol, 2011. **85**(12): p. 5970-4.
43. Fadda, L., et al., *HLA-Cw*0102-Restricted HIV-1 p24 Epitope Variants Can Modulate the Binding of the Inhibitory KIR2DL2 Receptor and Primary NK Cell Function.* PLoS Pathog, 2012. **8**(7): p. e1002805.
44. Apps, R., et al., *Influence of HLA-C expression level on HIV control.* Science, 2013. **340**(6128): p. 87-91.
45. Honda, K., et al., *Selection of escape mutant by HLA-C-restricted HIV-1 Pol-specific cytotoxic T lymphocytes carrying strong ability to suppress HIV-1 replication.* Eur J Immunol, 2011. **41**(1): p. 97-106.
46. Makadzange, A.T., et al., *Characterization of an HLA-C-restricted CTL response in chronic HIV infection.* Eur J Immunol, 2010. **40**(4): p. 1036-41.
47. Thomas, R., et al., *HLA-C cell surface expression and control of HIV/AIDS correlate with a variant upstream of HLA-C.* Nat Genet, 2009. **41**(12): p. 1290-4.
48. Kulkarni, S., et al., *Differential microRNA regulation of HLA-C expression and its association with HIV control.* Nature, 2011. **472**(7344): p. 495-8.
49. Apps, R., et al., *HIV-1 Vpu Mediates HLA-C Downregulation.* Cell Host Microbe, 2016. **19**(5): p. 686-95.
50. Adnan, S., et al., *Nef interference with HIV-1-specific CTL antiviral activity is epitope specific.* Blood, 2006. **108**(10): p. 3414-9.
51. Van Teijlingen, N.H., et al., *Sequence variations in HIV-1 p24 Gag-derived epitopes can alter binding of KIR2DL2 to HLA-C*03: 04 and modulate primary natural killer cell function.* AIDS, 2014.
52. Seich Al Basatena, N.K., et al., *KIR2DL2 enhances protective and detrimental HLA class I-mediated immunity in chronic viral infection.* PLoS Pathog, 2011. **7**(10): p. e1002270.
53. Ljunggren, H.G., et al., *Empty MHC class I molecules come out in the cold.* Nature, 1990. **346**(6283): p. 476-80.
54. Akari, H., et al., *Nef-induced major histocompatibility complex class I down-regulation is functionally dissociated from its virion incorporation, enhancement of viral infectivity, and CD4 down-regulation.* J Virol, 2000. **74**(6): p. 2907-12.
55. Fujiwara, M., et al., *Patterns of cytokine production in human immunodeficiency virus type 1 (HIV-1)-specific human CD8+ T cells after stimulation with HIV-1-infected CD4+ T cells.* J Virol, 2005. **79**(19): p. 12536-43.
56. Kitano, M., et al., *Identification and characterization of HLA-B*5401-restricted HIV-1-Nef and Pol-specific CTL epitopes.* Microbes Infect, 2008. **10**(7): p. 764-72.
57. Tomescu, C., et al., *Impact of protective killer inhibitory receptor/human leukocyte antigen genotypes on natural killer cell and T-cell function in HIV-1-infected controllers.* AIDS, 2012. **26**(15): p. 1869-78.
58. Ibe, M., et al., *Role of strong anchor residues in the effective binding of 10-mer and 11-mer peptides to HLA-A*2402 molecules.* Immunogenetics, 1996. **44**(4): p. 233-41.
59. Tanabe, M., et al., *Structural and functional analysis of monomorphic determinants recognized by monoclonal antibodies reacting with the HLA class I alpha 3 domain.* J Immunol, 1992. **148**(10): p. 3202-9.
60. Fan, Q.R., E.O. Long, and D.C. Wiley, *Crystal structure of the human natural killer cell inhibitory receptor KIR2DL1-HLA-Cw4 complex.* Nat Immunol, 2001. **2**(5): p. 452-60.

61. Dong, T., et al., *HIV-specific cytotoxic T cells from long-term survivors select a unique T cell receptor.* J Exp Med, 2004. **200**(12): p. 1547-57.
62. Miyashita, R., et al., *Association of killer cell immunoglobulin-like receptor genotypes with microscopic polyangiitis.* Arthritis Rheum, 2006. **54**(3): p. 992-7.
63. Hollenbach, J.A., et al., *Report from the killer immunoglobulin-like receptor (KIR) anthropology component of the 15th International Histocompatibility Workshop: worldwide variation in the KIR loci and further evidence for the co-evolution of KIR and HLA.* Tissue Antigens, 2010. **76**(1): p. 9-17.
64. Denis, L., et al., *Genetic diversity of KIR natural killer cell markers in populations from France, Guadeloupe, Finland, Senegal and Reunion.* Tissue Antigens, 2005. **66**(4): p. 267-76.
65. Qi, Y., et al., *KIR/HLA pleiotropism: protection against both HIV and opportunistic infections.* PLoS Pathog, 2006. **2**(8): p. e79.
66. Winter, C.C. and E.O. Long, *A single amino acid in the p58 killer cell inhibitory receptor controls the ability of natural killer cells to discriminate between the two groups of HLA-C allotypes.* J Immunol, 1997. **158**(9): p. 4026-8.
67. Winter, C.C., et al., *Direct binding and functional transfer of NK cell inhibitory receptors reveal novel patterns of HLA-C allotype recognition.* J Immunol, 1998. **161**(2): p. 571-7.
68. AK, M., et al., *Synergistic polymorphism at two positions distal to the ligand-binding site makes KIR2DL2 a stronger receptor for HLA-C than KIR2DL3.* J Immunol., 2008. **180**(6): p. 3969-3979.
69. Naruto, T., et al., *HLA class I-mediated control of HIV-1 in the Japanese population, in which the protective HLA-B*57 and HLA-B*27 alleles are absent.* J Virol, 2012. **86**(19): p. 10870-2.
70. Horton, H., et al., *Induction of human immunodeficiency virus type 1 (HIV-1)-specific T-cell responses in HIV vaccine trial participants who subsequently acquire HIV-1 infection.* J Virol, 2006. **80**(19): p. 9779-88.
71. Chikata, T., et al., *Host-specific adaptation of HIV-1 subtype B in the Japanese population.* J Virol, 2014. **88**(9): p. 4764-75.
72. Alter, G., et al., *Evolution of innate and adaptive effector cell functions during acute HIV-1 infection.* J Infect Dis, 2007. **195**(10): p. 1452-60.
73. Barbour, J.D., et al., *Synergy or independence? Deciphering the interaction of HLA Class I and NK cell KIR alleles in early HIV-1 disease progression.* PLoS Pathog, 2007. **3**(4): p. e43.
74. Long, B.R., et al., *Conferral of enhanced natural killer cell function by KIR3DS1 in early human immunodeficiency virus type 1 infection.* J Virol, 2008. **82**(10): p. 4785-92.
75. Alter, G., et al., *Differential natural killer cell-mediated inhibition of HIV-1 replication based on distinct KIR/HLA subtypes.* J Exp Med, 2007. **204**(12): p. 3027-36.
76. Moore, C.B., et al., *Evidence of HIV-1 adaptation to HLA-restricted immune responses at a population level.* Science, 2002. **296**(5572): p. 1439-43.
77. Brumme, Z.L., et al., *HLA-associated immune escape pathways in HIV-1 subtype B Gag, Pol and Nef proteins.* PLoS One, 2009. **4**(8): p. e6687.
78. Kawashima, Y., et al., *Adaptation of HIV-1 to human leukocyte antigen class I.* Nature, 2009. **458**(7238): p. 641-5.